EFFECTS OF SIMULTANEOUS OZONE AND NITROGEN EXPOSURE ON TWO TREE SPECIES: FAGUS SYLVATICA (L.) AND PICEA ABIES (L.) KARST.

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(Dekan)

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2. INTRODUCTION

In the eighties of the last century, the so called “new type forest decline” alarmed not only scientists, but also the public. Air pollution and acid deposition seemed to be responsible for a wide range of damages to forest trees. Since then, emissions have been of great concern, and a lot of efforts have been undertaken to reduce them. As a consequence, pollutants associated with heavy industry, such as sulphur dioxide could be reduced and thus only play a minor role in European forest health today. However, nitrogen deposition, especially from agricultural systems, and ozone concentrations do not appear to have declined in recent years and are therefore two of the most important pollution factors for forests in Europe (Bouwman et al., 2002; Matyssek and Innes, 1999). The main impacts of these pollutants on forest trees, which are of importance for this thesis, will be described briefly in the following chapters.

2.1 Ozone

Of the different types of air pollutants currently impacting forests in Europe, the greatest concern surrounds ozone (Matyssek and Innes, 1999). In the early fifties of the last century, smog was reported to cause injury to the leaves of certain plant species in southern California. Ozone, which was recognized as a phytotoxic pollutant already in 1956 (Middleton, 1956), was later on identified as the principal component of smog impacting plant health. Since then, it has received increasing recognition as an ecological problem. Analysis of historic ozone measurements revealed more than a doubling of tropospheric ozone concentration in the northern hemisphere during the last century. This increase was comprised between 1 and 2 % per year in the last two decades (Stockwell et al., 1997).

Tropospheric ozone is formed (and broken down) by various chemical reactions involving nitric oxides (NO_x) and atmospheric oxygen (O_2) under the influence of UV radiation (McKee, 1994); Figure 1). The presence of volatile organic compounds (VOCs) causes an accumulation of ozone. Oxidation of the photochemically active VOCs produces highly reactive chemicals, which convert NO to NO_2 without destruction of ozone. VOCs and NO_x, which derive mainly from fossil fuel combustion, can be transported long-distances, leading to high ozone concentrations in rural areas. Episodes of high ozone concentrations occur mainly in summer, being typically associated with periods of high barometric pressure, when atmospheric conditions are relatively stable.

a) Photodissociation of NO_2 by near-ultraviolet solar radiation ($280 \text{ nm} < \lambda < 430 \text{ nm}$)

$$\mathbf{NO_2 + radiation \rightarrow NO + O^{\cdot}}$$

b) Reaction of O and O_2 in the presence of M, which is a molecule (e.g. nitrogen) that removes excess energy of the reaction

$$\mathbf{O^{\cdot} + O_2 + M \rightarrow O_3 + M}$$

c) Absent any competing reactions, NO reacts rapidly with O_3

$$\mathbf{NO + O_3 \rightarrow NO_2 + O_2}$$

d) Resulting steady-state condition, with the constant depending on sunlight

$$\mathbf{[O_3] = constant\ [NO_2] / [NO]}$$

Figure 1: Chemical reaction of formation and breakdown of tropospheric ozone, after McKee (1994), slightly revised.

2.1.1 Critical levels of ozone

According to the UNECE (United Nations Economic Commission for Europe), critical levels are defined as the "concentrations of pollutants in the atmosphere above which direct adverse effects on receptors, such as plants, ecosystems, or materials, may occur according to present knowledge". The concept of critical levels for ozone was first defined at a workshop at Bad Harzburg, Germany, in 1988 (UNECE, 1988b), where values were expressed as a seasonal mean concentration. In following workshops, the critical levels for ground-level ozone have been developed further, leading to the definition of a cumulative exposure over a threshold concentration for plants: the AOT40 (accumulated ozone exposure over the threshold of 40 ppb). The accumulation time equals the vegetation period, which is from April to September in central Europe. The AOT40 is the basis for the so-called level I concept, to describe the general risk for adverse effects by ground-level ozone on plants. This exposure index proofed to be a useful indicator, though a flux-based risk assessment offers an improved quantitative evaluation (UNECE, 2003). The flux concept, the level II approach, takes into account factors modifying ozone uptake by the foliage, differences in ozone tolerance between species, spatial and temporal differences in phenology, and how ozone affects plant communities. Information on environmental conditions (photon flux density, temperature, vapour pressure deficit, and soil moisture deficit) are needed for calculation of this cumulative ozone uptake (Emberson et al., 2000a). However, at present, uncertainties in the development and application of the flux model for forests are still too large to justify their application as a standard risk assessment method at a European scale (Bull et al., 2003). In this thesis I refer to the presently used AOT40 model, but include the flux concept, where possible. For forests, the critical level was set at AOT40 5 ppm h (Karlsson et al., 2004), which is frequently exceeded in Swiss forests (Fuhrer et al., 1997). The calculated AOT40 levels for Switzerland are given in Figure 2.

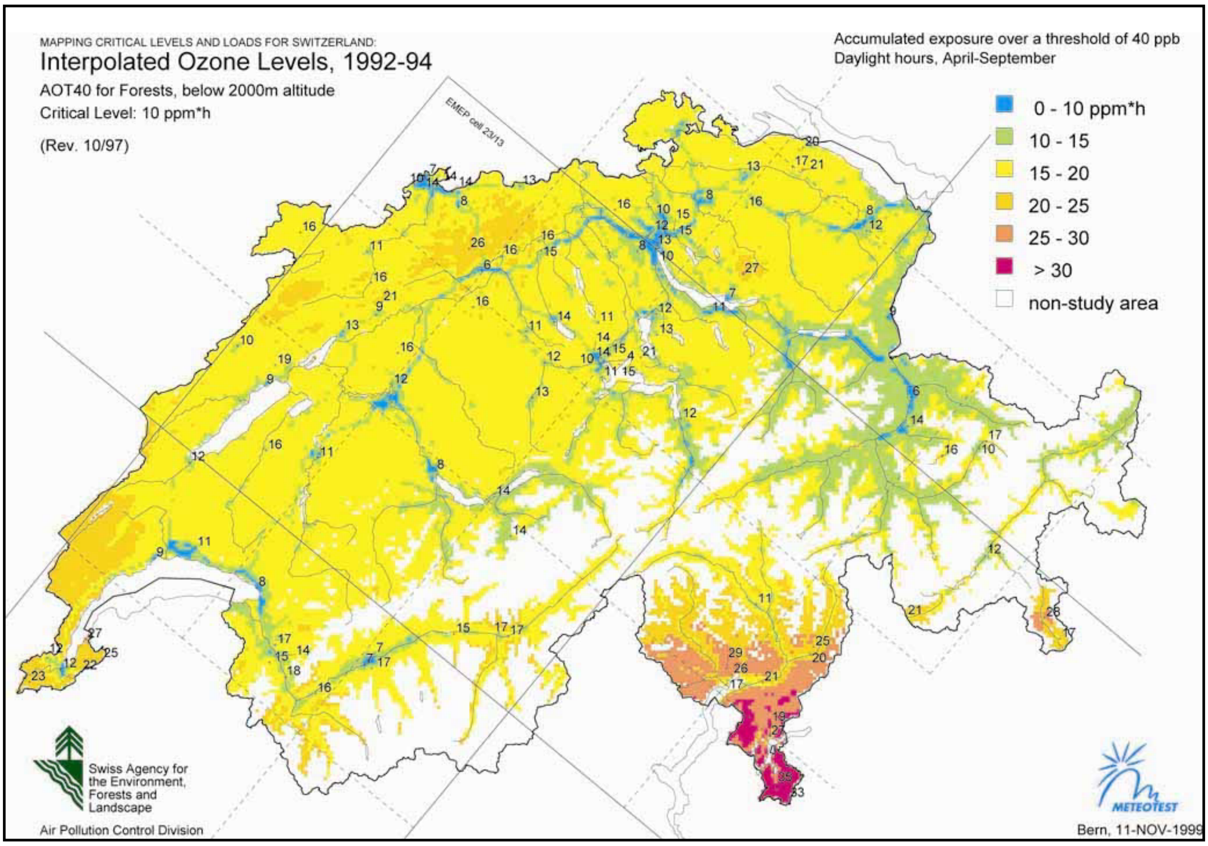

Figure 2: Interpolated AOT40 for forests below 2000 m altitude in Switzerland (1992 – 1994), based on 100 × 100 m grids. In this figure the outdated AOT40 of 10 ppm h is still used, as no update is yet available.

2.1.2 Visible symptoms

Most studies on the formation of visible injuries towards ozone exposure were conducted in the USA, but data on native European species are now also available (Novak et al., 2003). The first visible symptoms of ozone injury appear as interveinal light-green, red, brown, or purple stipples on the upper sides of sun-exposed leaves of broad-leafed tree species, or as the development of chlorotic mottling of needles in conifers (Skelly et al., 1999; Vollenweider et al., 2003). The injuries are caused by oxidative stress leading to cell necrosis in the assimilative tissue, which extends with increasing injury to the upper epidermis or to the lower palisade or spongy parenchyma layers (Vollenweider et al., 2003). Premature senescence and early shedding of leaves and needles is also frequently observed.

Tree species differ in their sensitivity towards ozone. Generally, broad-leafed tree species appear to be more sensitive, than conifers which can be explained by differences in leaf conductance and associated ozone uptake (Reich, 1987). But also genotypic variation can have great influence on sensitivity (Bortier et al., 2000b), which was shown for different tree species, e.g. beech seedlings (Paludan-Müller et al., 1999). The appearance of visible injuries is not the first damage that occurs, as other parameters, like photosynthesis, or carbon partitioning might be impaired much earlier.

2.1.3 Growth and biomass

One of the major impacts of ozone on trees is a reduction in height growth and biomass accumulation, which has been observed in many tree species. Here again, broad-leafed species like *Fagus sylvatica* appear to be more sensitive than conifers like *Picea abies* (Braun and Flückiger, 1995). A 10 % biomass reduction in *Fagus sylvatica* seedlings was observed over a time period of three years (AOT40 7 ppm h), whereas in spruce seedlings only a tendency towards a reduction could be stated. The reaction towards ozone exposure may be quite immediate, as tree seedlings fumigated for only one growing season with ozone (AOT40 1.72 ppm h), showed significant reductions in biomass (- 22 %) (Pääkkönen et al., 1996). These reductions in growth are often linked with the visible symptoms mentioned above.

In plants fumigated with ozone, less photosynthates are available for growth and biomass accumulation (Dizengremel, 2001). Furthermore, photosynthates are frequently needed for repair mechanisms and sometimes also for the production of new leaves to compensate for premature leaf loss. According to Skärby et al. (1998) a reduced CO_2 fixation, possibly without a concurrent decline in stomatal conductance, together with increased respiration rates cause a reduced carbon balance under ozone. The compensatory processes that maintain above-ground tissues might reduce root growth even stronger than that of shoots (Chappelka and Samuelson, 1998). This might be the main reason for the often observed increases in shoot : root ratios (Skärby et al., 1998).

2.1.4 Carbon allocation

Several studies describe ozone effects on sugar production and carbohydrate partitioning in trees (Andersen, 2003; Cooley and Manning, 1987). One explanation for the frequently observed decreases in storage carbohydrate concentrations is given by the reduced carbon balance mentioned above. Other authors found decreases of carbohydrate concentrations mainly in roots, whereas concentrations in aboveground plant parts, like needles or stems increased (Grulke et al., 2001; Lux et al., 1997). Dizengremel et al. (2001) suggested an impaired sucrose translocation leading to a modified partitioning of carbon above- and below-ground. It is thought that ozone fumigation impairs phloem loading and thus reduces carbon allocation to roots, whereas in aboveground plant parts carbohydrates accumulate (Cooley and Manning, 1987). This again is in accordance with the above-mentioned changes in growth, where roots are more affected than shoots. It is however possible, that increased sink strength of roots due to mycorrhizal colonization is sufficient to partially overcome the negative effects of ozone on allocation to roots and hence root : shoot balance (Mahoney et al., 1985).

2.1.5 Indirect effects

Changes in photosynthetic rates, carbohydrate production, C allocation, and C translocation are the key factors by which ozone influences tree growth and, ultimately, survival (Skärby et al., 1998). In addition to these effects of ozone on trees, further consequences have to be considered for tree health in the long-term. It has been shown that ozone increases the susceptibility towards drought, frost hardiness, parasites, and pathogens (Laurence and Andersen, 2003; Skärby et al., 1998). Also the combination with other pollutants could have severe consequences for forest trees.

2.1.6 Up-scaling from seedlings to mature trees

For logistical reasons, most studies on ozone used (potted) seedlings, in either indoor or outdoor chambers. Evidence has been provided, that chamber- and pot-effects might strongly influence or overlay the answers of tree seedlings towards treatments. For example Paludan-Müller et al. (1999) found that beech seedlings grown in open top chambers have 28 % more shoot biomass and 29 % less root biomass compared to seedlings grown outside, which was probably due to increased temperature and reduced light conditions inside the chambers. In addition, there is growing concern about whether ozone response data from seedlings can be extrapolated to mature trees (Kolb and Matyssek, 2001). Only few data are available for mature forest trees under ozone exposure. In some tree species (e. g. *Picea rubens*, *Pinus ponderosa*, *Prunus serotina*, *Sequoiadendron giganteum*) mature trees had lower stomatal conductance and lower ozone foliar injury compared to small trees (Kolb et al., 1997). Other studies suggest that mature trees might be even more susceptible towards ozone stress than seedlings. For example Samuelson and Edwards (1993) found, that photosynthesis of mature *Quercus rubra* was reduced by 25 % in ambient air, whereas no changes in seedlings occurred.

The installation of free-air ozone exposure experiments will greatly improve the knowledge on reactions of mature trees, although data for only a limited number of trees can be derived from these set ups (Matyssek and Innes, 1999; Tjoelker et al., 1995). The approach via pollution gradients could be very helpful for studies on ozone effects on mature trees. Experiments in California on mature Ponderosa pine along an ozone and nitrogen gradient have provided promising results in that context (Grulke et al., 2001), where significant reductions in root and needle starch concentrations were observed, with increasing pollutant concentrations.

2.2 Nitrogen

Nitrogen used to be the limiting factor for plant growth in many ecosystems. In the early 19th century estimated background nitrogen (N) inputs ranged between 1 and 3 kg N ha^{-1} yr^{-1}. Since then, but especially in the second half of the 20th century, the emissions of ammonia (NH_3) and nitric oxides (NO_x) have strongly increased in Europe. Nitric oxides derive mainly from fossil fuel combustion, whereas ammonia originates from intensive agricultural systems (Figure 3). These nitrogenous compounds are transported short and long distances leading to higher atmospheric nitrogen deposition in many natural and semi-natural ecosystems. For forests in Europe and the USA N deposition rates of 20 to 100 kg N ha^{-1} yr^{-1} have been observed (Bobbink et al., 2003).

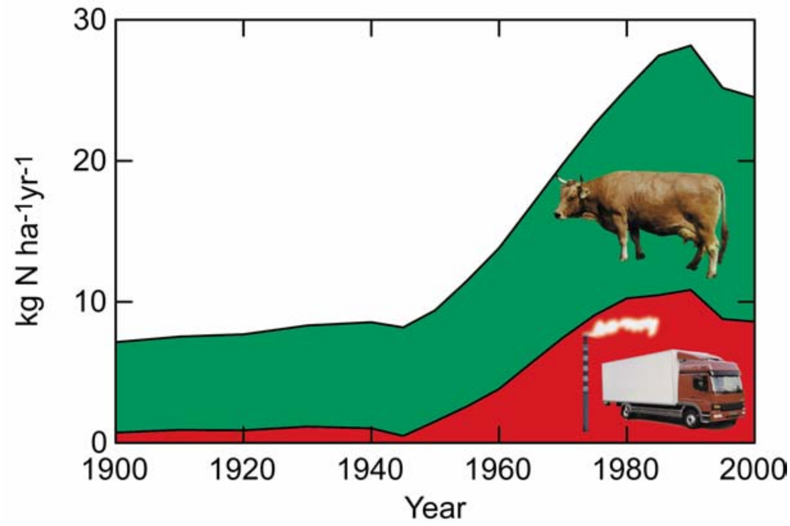

Figure 3: Historical development of nitrogen deposition in Switzerland (Flückiger and Braun, 2004), scaled after EKL (2004). Green: ammonia from agricultural systems; red: nitric oxides from fossil fuel combustion.

2.2.1 *Critical loads for nitrogen*

In Swiss forests the estimated nitrogen deposition rates reach levels of more than 40 kg N ha^{-1} yr^{-1} (EKL, 2004). At these deposition levels, nitrogen turns rather critical for natural ecosystems. Similar to ozone, critical loads for nitrogen deposition have been developed at several workshops. According to the UNECE, critical load is defined as “a quantitative estimate of an exposure to one or more pollutants below which significant harmful effects on specified sensitive elements of environment do not occur according to present knowledge” (UNECE, 1988a). During the last expert workshop on nitrogen in Berne, Switzerland (2002), a critical load for forests of 10 – 20 kg N ha^{-1} yr^{-1} was recommended (Bobbink et al., 2003), which is currently exceeded in about 90 % of Swiss forests (Rihm, 1996); Figure 4).

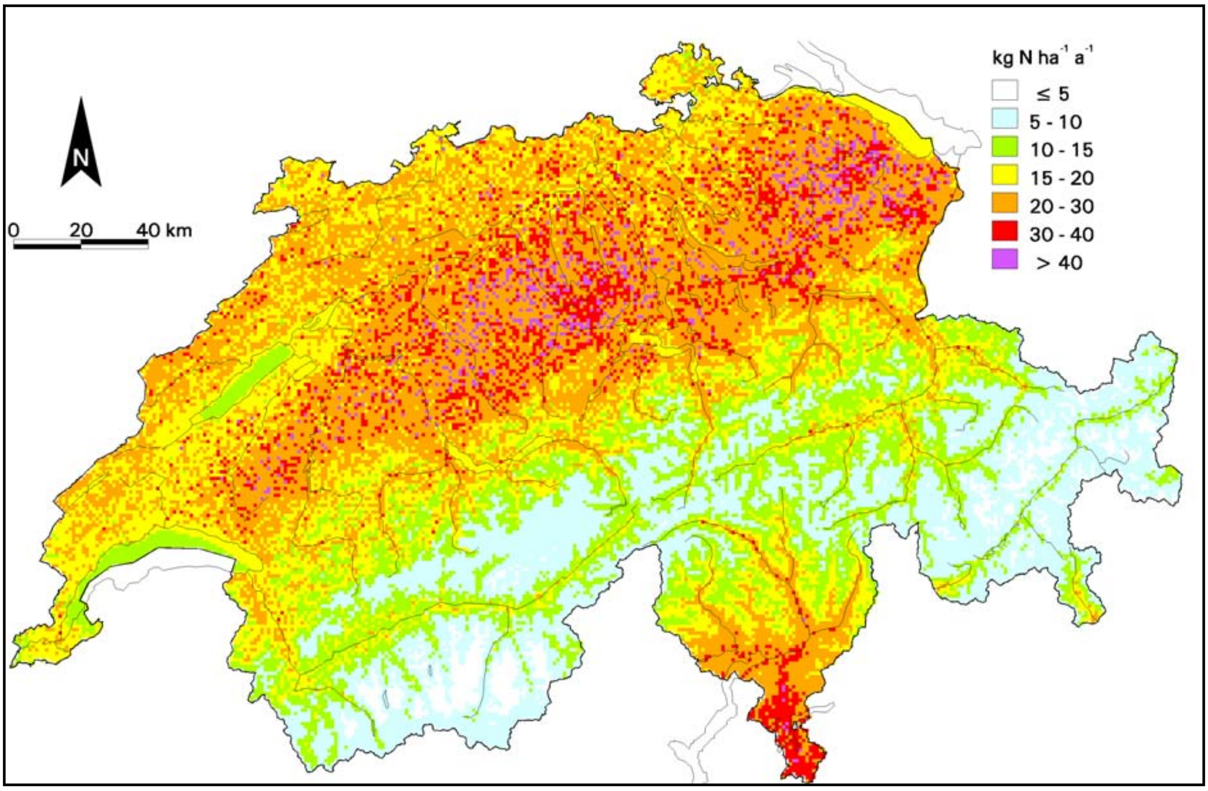

Figure 4: Modelled N-Deposition in Switzerland (EKL, 2004). Deposition was calculated for the year 2000.

2.2.2 Growth and biomass

The most obvious changes in trees caused by an increased nitrogen supply are fertilisation effects. As long as no other factors are limiting, nitrogen addition leads to enhanced shoot elongation and biomass accumulation. These positive influences are well documented for many different tree species, including *Picea abies*, *Fagus sylvatica*, *Betula pendula*, or *Pinus sylvestris* (Flückiger and Braun, 1999; Pääkkönen and Holopainen, 1995; Utriainen and Holopainen, 2001b). As a consequence forest growth increased in the last 40 – 50 years in temperate European regions (Spiecker et al., 1996). Despite these findings, recent data suggest, that chronic nitrogen deposition can reverse, or at least attenuate the initial positive effects (Boxman et al., 1998). For instance in southern Norway, a study on more than 31000 spruce trees revealed an increased growth until approximately 1970, with a following growth decline from 1980 onwards in plots with a modelled wet nitrogen deposition of more than 15 kg N ha^{-1} yr^{-1} (Nellemann and Thomsen, 2001). In plots with a modelled wet deposition of 7 – 15 kg N ha^{-1} yr^{-1} growth started to decrease in the 1990s.

2.2.3 Roots and mycorrhiza

The nitrogen induced growth enhancements refer mainly to aboveground plant parts, whereas root growth does not necessarily increase. In ecosystems which are not nitrogen saturated a stimulation of root growth might occur (Persson and Ahlström, 1990), but other publications suggest rather a reduction in fine root biomass and root length when nitrogen deposition is enhanced (Matzner and Murach, 1995). Combined with the frequently detected increases in aboveground biomass, an increase in the shoot : root ratio is the consequence (Flückiger and Braun, 1998; Pääkkönen and Holopainen, 1995). Additionally, other alterations in fine roots will probably also influence the mycorrhization of trees. For instance, the ramification of roots providing short roots, which can be colonized by mycorrhizal fungi, can be drastically reduced by fertilisation (Ahlström et al., 1988). Negative impacts on the fruit body production, the production and distribution of the extraradical mycelium in the soil, and the formation of ectomycorrhizas under different nitrogen regimes have been reported (Wallenda and Kottke, 1998).

2.2.4 Carbon allocation

The observed increases in shoot growth require an enhanced utilisation of photosynthates, which leads to a lowered availability of carbohydrates for storage (von Fircks and Sennerby-Forsse, 1998). Wallenda et al. (1996) proposed a higher need for carbon skeletons for amino acid synthesis in *Picea abies* under increased inorganic nitrogen supply, also resulting in less storage carbohydrates. These hypotheses are supported by findings of Grulke et al. (2001), who showed decreasing monosaccharide and starch concentrations in needles and roots of mature *Pinus ponderosa* trees under enhanced nitrogen deposition (up to 20 – 40 kg N ha^{-1} yr^{-1}) in California. In this case however, ozone concentrations were also enhanced together with increasing nitrogen deposition.

2.2.5 Nutrient concentrations and nutrient ratios

Reports on changes of nutrient concentrations and nutrient ratios in leaves are quite numerous for trees fertilised with nitrogen. In the majority of cases, nitrogen concentrations in leaves or needles increase, whereas phosphorus concentrations decline (Balsberg Påhlsson, 1992; Flückiger and Braun, 1999). Concentrations of potassium, calcium, magnesium, or other nutrients do not always show the same reaction, leading to decreases or no changes, which might depend on soil properties or plant species (Lippert et al., 1996; Utriainen and Holopainen, 2001b). A likely explanation for the observed effects on nutrient concentrations is an impact on the mycorrhizal fungi. As mentioned above nitrogen fertilisation might reduce mycorrhization and will thus certainly influence the uptake of nutrients. Another approach was made by Haynes (1982), who suggested a reduced uptake of phosphorus as a result of soil acidification and hence aluminium toxicity, caused by competition with NH_4^+ in the soil. But also a decreased supply rate of nutrients other than nitrogen due to leaching and nitrogen induced high growth rates depleting the resource have been suggested (Thelin et al., 1998).

As a consequence of the observed changes in the nutrient concentrations, the nutrient ratios are also affected. Especially the N : P ratio is very likely to increase, but also changes in other nutrient ratios, like N : K, N : Mg, or N : Ca have been observed (Flückiger and Braun, 1999; Seith et al., 1996).

2.2.6 Soil acidification

Even though accelerated soil acidification plays only a minor role in this thesis it is a very important factor in the context of increased nitrogen deposition.

Soil acidification is a naturally occurring but very slow process. However, it may be accelerated by enhanced nitrogen input. Nitrogen is deposited in oxidised or reduced forms, e.g. as nitrate or ammonium. The latter is either taken up by plant roots or subjected to nitrification yielding nitrate. Both processes result in proton release into the soil. If nitrate, either from deposition or nitrification, is not taken up by plant roots, it will easily be leached out together with base cations, leaving protons with the consequence of soil acidification (Fangmeier et al., 1994). Leaching of base cations such as potassium, calcium, or magnesium and an enhanced dissolution of aluminium have been observed in poorly buffered soils (van Breemen and van Dijk, 1988). An increased aluminium release has been shown to reduce fine root development and mycorrhiza, which will lead to reduced nutrient uptake (Persson and Majdi, 1995).

2.2.7 Indirect effects

Similar to ozone, nitrogen deposition has several indirect effects on tree health in the long-term. An enhanced nitrogen input, leading to eutrophication, might render trees more susceptible to drought, parasite and pathogen attacks, and also to windfall due to decreased stability (Braun et al., 2003; Fangmeier et al., 1994; Jones et al., 2004). Additionally the combination with other pollutants, like ozone or enhanced CO_2 concentrations could have severe consequences for forest tree health.

2.3 Combination of nitrogen and ozone

Even though many studies deal with nitrogen (N) or ozone (O_3) influence on forest trees, the combinatory effects of the two pollutants are only partly understood. Investigations of simultaneous O_3 and N impacts are of specific significance, as both pollutants might affect similar variables, like growth, biomass allocation, or carbohydrate concentrations in various plant organs. In addition, already today, both pollutants frequently exceed the critical loads/levels and are likely to increase even further in the future. To assess the overall impact of these pollutants on forest stands it is not sufficient, although necessary, to conduct experiments in controlled conditions with seedlings submitted to N or O_3 alone (Dizengremel, 2001). This is even more so as generation of ozone is coupled to the existence of nitric oxides (Figure 1). The importance of studying effects of concurrent N and O_3 exposure has been recognised and in recent years, although still few, an increasing number of studies have focused on this issue.

For example, in a study along an O_3 and N gradient Grulke et al. (2001) have shown that carbon partitioning of mature *Pinus ponderosa* was changed under simultaneously elevated O_3 and N exposure: carbohydrate concentrations in roots and needles decreased, whereas bole carbohydrate concentrations increased (80 ppb of O_3 hourly average; 20 – 40 kg N ha^{-1} yr^{-1}). Scots pine seedlings reacted to chronic ozone exposure ($AOT40_{dl}$ 2.8 – 9.4 ppm h) with strongest growth reductions when combined with high nitrogen availability (Utriainen and Holopainen, 2001b). Adversely, no negative ozone effects (AOT40 18.0 and 9.2 ppm h) on leaf yellowing or earlier senescence were observed for *Betula pendula* seedlings grown under high nitrogen supply (74 and 150 kg N ha^{-1} yr^{-1}) (Pääkkönen and Holopainen, 1995). The authors concluded that nitrogen might confer birch seedlings with greater resistance to ozone. In seedlings of *Picea abies*, nitrogen fertilisation did not affect the influence of ozone ($AOT40_{dl}$ 6.9 ppm h) in terms of growth or biomass partitioning (Utriainen and Holopainen, 2001a).

The scarcity of information in general, the heterogeneity of results, and a missing evaluation of the applicability of up-scaling from seedlings to mature trees stress the need for further studies in order to be able to assess the overall impact of the two pollutants on forest stands.

2.4 Aims of the study

The general goal of my studies was to contribute to a far more precise assessment of the impact of the two major pollutants in central Europe, tropospheric ozone (O_3) and nitrogen deposition (N), on tree health.

In more detail, I was interested in the following questions:

- How do forest trees react to a simultaneous exposure of O_3 and N?
  Disregarding potential interactions of the effects a concurrent exposure to both pollutants will affect different ecophysiological parameters and in last consequence tree health. These changes might be attributed to O_3 or N exposure or a combination of both.
- Do the effects of O_3 and N on forest trees interact?
  Similar parameters might be affected by either N or O_3 exposure, e.g. shoot elongation,

biomass accumulation, or root carbohydrate concentrations. Regarding these parameters trees might react to concurrent exposure in a simple additive way, i.e. positive and/or negative trends sum up to a total effect. In this case individual effects do not interact. Adversely, O_3 and N effects interact, which may lead to either aggravation (synergy) or alleviation (antagony) of the sum of individual effects.

- Is it legitimate to up-scale results gained from seedling experiments to mature trees?
  Numerous studies added valuable contribution to our understanding of the influence of O_3 and/or N on tree seedlings. But may we confer this knowledge to mature trees? Specifically, comparable literature on O_3 effects on seedling and mature trees is scarce. But at least some of the rare evidence indicate, that mature trees might be even more susceptible towards ozone stress than seedlings.
- Are there interspecific differences in the reaction towards O_3 and N?
  Since primary targets of N fertilisation and O_3 exposure should be the same in different tree species differences are expected to be qualitatively similar but may vary quantitatively. The sensitivity to the generally fertilising effect of increasing N deposition and the impact of O_3 on mycorrhiza may play a key role in the context of interspecific differences.

Using experimental and comparative field studies as one approach Switzerland is ideal, because models are available that calculate both tropospheric ozone concentrations as well as nitrogen depositions covering the entire country. Furthermore, according to these models and numerous measurements critical levels/loads are exceeded frequently in Switzerland.

To be able to gain results as meaningful as possible I chose the two main tree species of Swiss forests: *Fagus sylvatica* and *Picea abies*.

Two approaches were chosen to clarify the above raised questions:

The first approach was a three-year fumigation and fertilisation experiment with seedlings of the two tree species. Combinatory O_3 and N effects were monitored on whole-plant level (above- and belowground biomass) as well as on compartment level (roots, stems, needles, or leaves). Additionally, I observed and evaluated needle discolorations in *Picea abies* and leaf necroses and aphid infestations in *Fagus sylvatica*. The results of this experiment are described and discussed in chapter 3 (*Picea abies*) and chapter 4 (*Fagus sylvatica*).

In a second approach I conducted a gradient study with mature trees of *Fagus sylvatica* and *Picea abies*. Twenty sites for beech and 21 sites for spruce were chosen along a gradient of N deposition and O_3 concentrations in Switzerland. All sites had acidic soil conditions and elevation was not higher than 1100 m asl. Roots were excavated to test for O_3 and N effects on carbohydrates concentrations. The results of the starch analysis are presented in chapter 5 (beech) and 6 (spruce), whereas the results of the soluble carbohydrate analysis are given in the appendix (chapter 10).

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3. EFFECTS OF SIMULTANEOUS OZONE EXPOSURE AND NITROGEN LOADS ON CARBOHYDRATE CONCENTRATIONS, BIOMASS, AND GROWTH OF YOUNG SPRUCE TREES (*PICEA ABIES* [L.] KARST.)

3.1 Abstract

Spruce saplings were grown under different nitrogen fertilisation regimes in eight chamberless fumigation systems, which were fumigated with either charcoal-filtered (F) or ambient air (O_3). After the third growing season trees were harvested for biomass and non-structural carbohydrate analysis. Nitrogen had an overall positive effect on the investigated plant parameters, resulting in increased shoot elongation, biomass production, fine root soluble carbohydrate concentrations, and also slightly increased starch concentrations of stems and roots. Only needle starch concentrations and fine root sugar alcohol concentrations were decreased. Ozone fumigation resulted in needle discolorations and affected most parameters negatively, including decreased shoot elongation and decreased starch concentrations in roots, stems, and needles. In fine roots however, soluble carbohydrate concentrations remained unaffected or increased by ozone fumigation. The only significant interaction was an antagonistic effect on root starch concentrations, where higher nitrogen levels alleviated the negative impact of ozone.

3.2 Introduction

The impacts of ozone exposure or nitrogen deposition on forest trees have been examined widely but few studies take into account the combination of the two factors. In recent decades, ambient ozone doses often exceeded the critical level of 5 ppm h AOT40 daylight (accumulated exposure over a threshold of 40 ppb) (Karlsson et al., 2004). Ozone doses above these levels can cause various effects on different tree species: visible injuries, growth reductions, shifts in shoot : root biomass ratio, and also impacts on physiological processes have been reported (Braun and Flückiger, 1995; Fuhrer et al., 1997; Ottoson et al., 2003; Wallin et al., 1996). Decreases in assimilation rate (Grulke et al., 2002) and increases in respiration (Willenbrink and Schatten, 1993) can lead to less carbon fixation. It is thought that ozone can restrict phloem loading und thus assimilate translocation from shoots to roots (Skärby et al., 1998). In addition, Bortier et al. (2000b) suggested carbon retention in the leaves for repair of damaged foliage. This results in less storage compounds like starch or soluble carbohydrates especially in roots (Braun et al., 2004; Lux et al., 1997). Nitrogen deposition has also increased during the second half of the last century, exceeding the critical N loads for forests of 10 – 20 kg N ha^{-1} yr^{-1} (Achermann and Bobbink, 2003) in about 90 % of

Swiss forest by now (Rihm, 1996). Nitrogen is known to increase growth and biomass production, especially in aboveground plant parts (Kainulainen et al., 2000), also resulting in changes of the shoot : root ratios (Lippert et al., 1996). The enhanced shoot growth leads to increased utilisation of photosynthate and lower carbohydrate availability for storage (von Fircks and Sennerby-Forsse, 1998). According to Wallenda et al. (1996) a higher need for carbon skeletons for amino acid synthesis under increased inorganic nitrogen supply is also likely, which would result in less storage carbohydrates.

The combination of the two pollution factors will therefore have significant impacts, especially on carbon allocation and carbohydrate storage of trees. So far, few studies have examined the simultaneous effects of nitrogen fertilisation and ozone fumigation. Grulke et al. (2002) found significant decreases in root starch concentrations of mature *Pinus ponderosa* trees under high nitrogen and ozone levels. Thomas et al. (2002) found similar results for the root starch concentrations of mature beech, but for high ozone and nitrogen pollution, the two factors seemed to alleviate the effect of each other. Other studies indicated that young trees might be less responsive to ozone under nitrogen fertilisation, in terms of biomass accumulation (Lippert et al., 1996; Pell et al., 1995). In our three-year study we examined the combination of ozone fumigation and nitrogen fertilisation on growth, biomass production, and carbohydrate storage of spruce saplings and possible interactions of the two pollution factors. We hypothesized mainly synergistic negative impacts, especially on sensitive parameters like carbohydrate concentrations.

3.3 Materials and Methods

3.3.1 Plants

Two-year old Norway spruce saplings from a local provenance (400 m altitude) were planted together with beech seedlings into the bare soil within eight units (see below) in March 2000. The soil was a calcium-rich Luvisol, with former agricultural use and pH ($CaCl_2$) values between 5.2 and 7.1. The plants were watered with tap water in conditions of dry weather. Results of the beech trees are not mentioned here, but will be published in another paper.

3.3.2 Fumigation system

Chamberless fumigation units were constructed according to Leyendecker et al. (1996) at the research site in Schönenbuch, Switzerland (latitude 47.5 °, altitude 400 m asl). They consisted of two concentric rings of perforated plastic tubes (tube diameter: 9.5 cm). The outer fumigation ring had a diameter of 3 m and was perforated on one side towards the centre, the inner ring with a diameter of 1.2 m, was perforated on two sides and consisted of two tubes.

The rings were surrounded by a plastic enclosure of 80 cm height to protect from wind. Air was blown through the system at 13 m^3 min^{-1} either directly from outside or through activated charcoal filters (C1, Gertsch Filter, Balsthal, Switzerland), with the blowers switched off at night (solar irradiation < 50 W m^{-2}). This setup minimized the climatic effects usually encountered with open-top chambers. The average temperature increase in the fumigation units was 0.4 °C, with a 95 % tile of 2.6 °C.

Each treatment consisted of four units. The units with outside air were enriched with ozone generated with a Fischer 500M ozone generator (Fischer technology, Bonn, Germany) from pure oxygen to compensate for the system loss. Outside concentrations and concentrations within the units were measured using a Dasibi 1008 AH ozone monitor (Dasibi, Glendale, CA, USA) which was calibrated once a year with an EPA traceable transfer standard. The gas input was switched between the units using a Gas Tracer Surveyor (Icam, Worthing, UK). Ozone loss through the tubing from the units to the monitor ranged between 10 and 15 % and was corrected for. The ozone data from April to September of each experimental year are given in Table 1. Average concentrations and AOT40 (Fuhrer 1997) were calculated during daylight hours. A cumulative stomatal uptake for Norway spruce was computed according to Emberson et al. (2000b), with modifications as described by Karlsson et al. (2004) using either no threshold or a threshold of 1.6 nmol m^{-2} s^{-1}. The meteorological parameters needed for the flux calculation were measured on site: global radiation using a Kipp solarimeter, air temperature and humidity using an aspiration psychrometer (both Friedrichs, Schenefeld, Germany). Wind speed was assumed to be non-limiting because of the air flow inside the units. Soil moisture was monitored with an Equitensiometer EQ2 (Ecomatik, Dachau, Germany) at a depth of 20 cm. The values were below -0.05 MPa, the upper level for stomatal closure assumed in the flux model, during 10 % of the time, with an estimated maximum conductance reduction by 29 %.

Table 1: Ozone data during the 3 year experimental period between April and September (average of the four fumigation units ± standard deviation).

		Mean daylight hours [ppb]	AOT40 daylight hours [ppm h]	Cumulative ozone uptake threshold zero [mmol m^{-2} proj. area]	Cumulative ozone uptake threshold 1.6 nmol m^{-2} s^{-1} [mmol m^{-2} proj. area]
2000	filtered	19.4 ± 0.6	0.1 ± 0.0	2593 ± 101	0.4 ± 0.1
2000	ambient	37.6 ± 3.1	11.5 ± 4.6	4871 ± 426	5.9 ± 1.4
2001	filtered	22.1 ± 2.2	0.5 ± 0.4	2084 ± 186	0.4 ± 0.1
2001	ambient	35.2 ± 5.7	7.2 ± 5.3	3177 ± 555	2.0 ± 1.3
2002	filtered	28.3 ± 3.5	2.7 ± 2.5	3945 ± 427	3.4 ± 1.3
2002	ambient	47.4 ± 0.2	22.1 ± 0.1	6458 ± 90	11.5 ± 0.3

3.3.3 Nitrogen treatment

Each fumigation unit was divided into quarters for different nitrogen fertilisation. Plastic foil was used to separate the quarters and the whole units from the surrounding soil to a depth of 70 cm. The quarters received liquid nitrogen fertilisation (NH_4NO_3) of 0, 20, 40, and 80 kg ha^{-1} yr^{-1} in six monthly treatments during the growing seasons (April – September) in 2000, 2001, and 2002. Modelled background N deposition was 15 kg N ha^{-1} yr^{-1}. Seventy percent of this N input is in gaseous form (NH_3 (4.1 µg m^{-3}), NO_2, and HNO_3), which may have been reduced by the filtration.

3.3.4 Morphological measurements

Annual shoot elongation was measured each year after the growing season. Needle discolorations were first observed in summer 2001 and developed further during the growing season of 2002. In September 2002 they were evaluated using ten different categories from no to strong injuries (0 - 9).

3.3.5 Soil-pH

Soil-pH was measured every year in October, approximately four weeks after the last fertilisation. In each unit, five soil samples were taken with a soil-drill (diameter: 1.0 cm) from the soil surface to a depth of 20 cm. Samples were dried at 40 °C for two days and pH was measured electrometrically in a 1 : 2.5 suspension with $CaCl_2$-solution (0.01 M) (713 pH Meter, Metrohm, Herisau, Switzerland).

3.3.6 Collection of Plant material

After the third growth period (winter 2002/03), eight spruce trees per quarter were harvested destructively for biomass determination, soluble carbohydrate analysis of fine roots (diameter: ≤ 0.7 mm), and starch analysis of medium sized roots (diameter: > 1.0 to ≤ 2.5 mm), current stems, and current needles. Roots were cleaned from adhering soil, separated in fractions, shock frozen in liquid nitrogen, lyophilised, and ball-milled, as described in detail by Thomas et al. (2002). Aboveground plant parts were washed carefully with cold water. Current shoots were separated, rinsed with deionised water and treated like the root samples. After lyophilisation, spruce twigs and needles were separated. Needles were also used for nutrition analysis as described below. The remaining above- and belowground plant material was oven dried for at least 2 days at 70 °C and weighed for biomass determination.

3.3.7 Nutrient analysis

After preparation as described above, three needle samples of each fertilisation treatment within each unit, were digested with H_2SO_4/H_2O_2 and a $Se/LiSO_4$ catalyst, following Allen (1989). Nitrogen and phosphorus were measured colorimetrically (Walinga et al., 1995) whereas Ca, K, Mg, and Mn were analysed with an AAS (SpectrAA-10, Varian, IL, USA). Apple leaves (National Institute of Standards and Technology, Gaithersburg, MD, USA) were used as a standard.

3.3.8 Carbohydrate analysis

Soluble carbohydrate analysis of the fine roots was performed after the method of Peuke et al. (2002) with an ion chromatograph, equipped with a CarboPac PA10 column plus guard column and an ED50 electrochemical detector (Dionex Corporation, Sunnyvale, CA, USA). The duration of a run was 80 minutes with a gradient of NaOH from 16 mM to 200 mM. The following carbohydrates were detected: sugar alcohols: arabitol, mannitol, pinitol, and sorbitol; monosaccharides: arabinose, fucose, fructose, galactose, glucose, mannoheptulose, mannose, and rhamnose; di- and trisaccharides: maltose, raffinose, sucrose, and trehalose.

Pinitol and myo-inositol could not be separated and the peak was treated as pinitol only, as this is one of the major sugar alcohols in spruce (Lux et al., 1997). Xylose and mannose could also not be separated and were treated as mannose only, which occurs in higher concentrations than xylose in spruce (Lux et al., 1997).

The starch concentrations of the roots, twigs, and needles were measured chromatographically as glucose units. Twenty mg of the dried samples were mixed with 40 mg of insoluble polyvinylpyrrolidone (Polyclar AT, Serva, Heidelberg, Germany) to remove phenolic compounds during extraction. Other soluble compounds were eliminated by washing the samples three times with 1 ml of deionised water. One ml citrate-buffer solution (pH 4.6) and 20 µl of amyloglucosidase from *Aspergillus niger* (33 U) (Boehringer, Mannheim, Germany) were added and the vials were placed in a heated shaker (60 °C) for one hour (HTM 130, Haep Labor Consult, Bovenden, Germany). After cooling and centrifugation (Centrifuge 5415

D, Vaudaux-Eppendorf, Hamburg, Germany; 6000 *g*, 15 min), the supernatants were filtered (RC55 membrane filters 0.45 µm, Schleicher and Schuell, Dassel, Germany) and stored in a freezer (- 20 °C) until injection into the same chromatographic system as used for soluble carbohydrates. Depending on the starch concentrations different dilutions were used for the different plant parts (roots: 100 x, stems: 10 – 50 x, needles: 5 – 50 x). A CarboPac PA10 column plus guard column with 160 mM NaOH (isocratic) as the mobile phase were used.

3.3.9 Statistics

For statistical analysis SYSTAT 10 (Systat Inc., Evanston, IL, USA) and S-PLUS 2000 (MathSOFT INC., Cambridge, MA, USA) were used. Shoot length, starch, and soluble carbohydrate concentrations were root-transformed and starch concentrations of the needles were ranked before analysis. Sugars were combined to groups and tested as such (alcohols, monosaccharides, di- and trisaccharides, and total). In order to reduce the effects of gradients in soil conditions and light within the experimental field, blocks were formed which consisted of one filtered and one ambient air unit each (Schuster and von Lochow, 1991) and served as a random grouping variable in the statistical analysis (S-PLUS, linear mixed effects model). A confounding pH gradient remained nevertheless which necessitated its consideration as a covariate. The residuals of the tests were checked for normal distribution and the presence of outliers.

3.4 Results

3.4.1 Visible symptoms

In the second growth period some spruce saplings started to show visible symptoms of the ozone fumigation. Needles of the shoot tips became yellow and developed chlorotic spots in the third growth period. The discoloration was evaluated in the third growth period showing a significant positive correlation with ozone fumigation ($p < 0.001$) and also with nitrogen fertilisation ($p < 0.001$) (Figure 1), but no interaction could be detected.

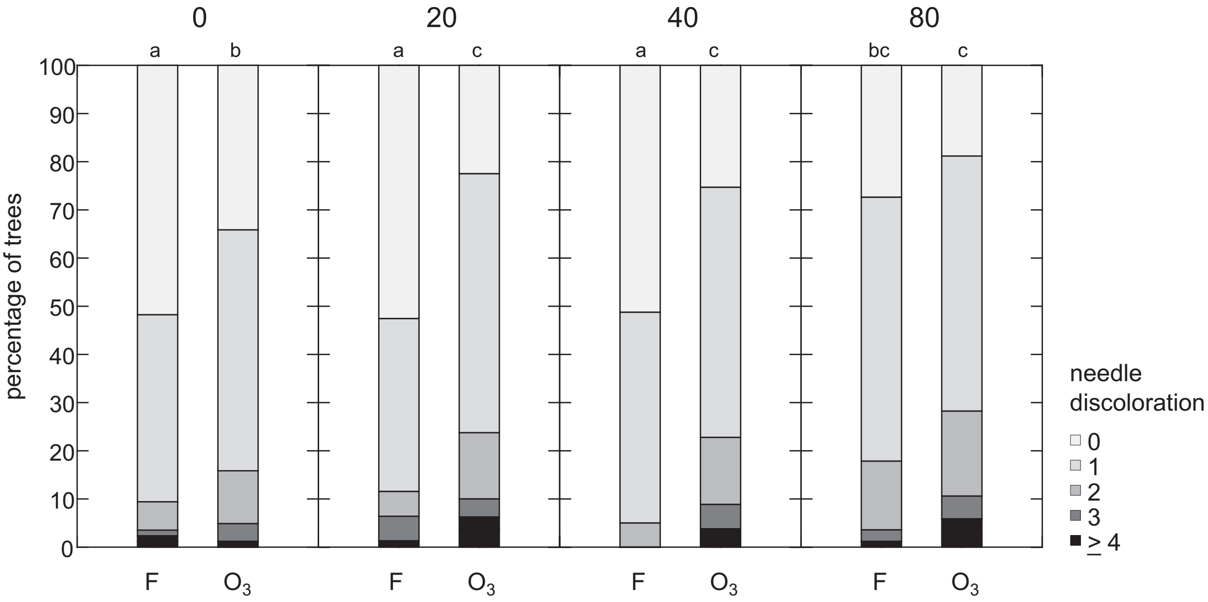

Figure 1: Percentage of spruce trees showing needle discolorations with different nitrogen fertilisation treatments and in filtered (F) or ambient air (O_3). Nitrogen fertilisation [kg N ha^{-1} yr^{-1}]: 0, 20, 40, 80. Ozone ($p < 0.001$) and nitrogen ($p < 0.001$) have significant effects on visible symptoms, without interaction of the two pollutants. Differences ($p < 0.05$) are indicated by different letters.

3.4.2 Soil-pH

The soil pH of the 80 kg N ha^{-1} yr^{-1} treatment started to decline slightly over the time of the experiment, which was detected as a significant increase in the pH-difference between the years 2000 and 2002 ($\Delta pH = 0.022$; $p = 0.042$) (data not shown). For the other treatments no differences were found.

3.4.3 Elongation and biomass

Ambient ozone had a negative effect on annual shoot elongation from the year 2001 onwards, but growth was significantly enhanced by nitrogen fertilisation, showing no significant interaction between the two pollutants (Table 2). Impact of nitrogen fertilisation and ozone fumigation on shoot elongation of the second and third growth period are shown in Figure 2. For total above- or belowground biomass no influence of the ozone fumigation could be detected. Nitrogen fertilisation enhanced biomass accumulation significantly in the aboveground as well as in the belowground plant parts. The shoot : root ratio was significantly reduced by the ozone fumigation. Nitrogen had no effect on the shoot : root ratio in any of the harvest years and no interaction of the two pollutants could be detected (Table 2).

Table 2: Effect of ozone fumigation (O_3) and nitrogen fertilisation (N) on annual shoot elongation, biomass production, and shoot: root dry mass ratio of *Picea abies*. Data were corrected for soil pH. Values are means ± SE; n = 256 observations in 4 groups; mixed effects linear model: (*) = $p < 0.1$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$; ns = not significant.

treatment	**shoot elongation** [cm]			**total biomass** [g]		
	2000	**2001**	**2002**	**shoots**	**roots**	**shoot : root**
filtered						
0 [kg N ha^{-1} yr^{-1}]	4.24 ± 0.38	10.97 ± 0.63	16.62 ± 0.53	30.76 ± 2.03	11.63 ± 0.82	2.71 ± 0.09
20 [kg N ha^{-1} yr^{-1}]	4.09 ± 0.36	9.84 ± 0.54	16.22 ± 0.42	30.80 ± 2.66	11.64 ± 1.01	2.74 ± 0.08
40 [kg N ha^{-1} yr^{-1}]	4.26 ± 0.29	11.15 ± 0.68	19.01 ± 0.53	36.91 ± 3.75	14.06 ± 1.58	2.75 ± 0.10
80 [kg N ha^{-1} yr^{-1}]	4.20 ± 0.28	13.02 ± 0.83	17.94 ± 0.71	43.81 ± 2.81	17.28 ± 1.43	2.74 ± 0.12
ambient						
0 [kg N ha^{-1} yr^{-1}]	3.87 ± 0.26	8.73 ± 0.62	13.86 ± 0.70	29.43 ± 2.18	13.50 ± 1.19	2.33 ± 0.09
20 [kg N ha^{-1} yr^{-1}]	3.51 ± 0.20	8.34 ± 0.53	13.16 ± 0.73	28.22 ± 2.56	12.16 ± 1.11	2.34 ± 0.11
40 [kg N ha^{-1} yr^{-1}]	4.10 ± 0.32	10.64 ± 0.68	15.88 ± 0.61	34.73 ± 2.28	13.62 ± 1.02	2.75 ± 0.10
80 [kg N ha^{-1} yr^{-1}]	4.67 ± 0.47	10.47 ± 0.63	15.93 ± 0.71	44.29 ± 3.49	18.81 ± 1.48	2.40 ± 0.06
O_3	ns	***	***	ns	ns	***
N	ns	**	**	***	***	ns
O_3*N	ns	ns	ns	ns	ns	ns

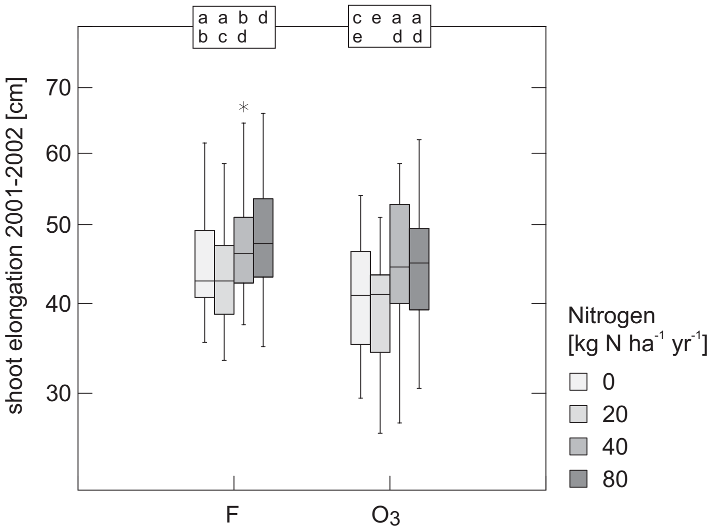

Figure 2: Effects of ozone fumigation and different nitrogen fertilisation treatments on shoot elongations of spruce trees of two consecutive years (2001 and 2002). F: filtered air units, O_3: ambient air units. Ozone ($p < 0.001$) and nitrogen ($p < 0.001$) have significant effects on shoot elongation, without interaction of the two pollutants. Differences ($p < 0.05$) are indicated by different letters.

3.4.4 Starch concentrations

Starch concentrations of the roots, stems, and needles were significantly reduced by ozone fumigation (Table 3). Nitrogen had a slightly positive effect on the starch concentration in roots, especially in ambient air. In the stems, an overall slight increase of starch concentrations with increasing nitrogen was observed. Deviations from the general trend were, however, quite substantial. Needle starch concentrations showed a significant negative relation with nitrogen fertilisation except for the treatment with 80 kg N ha^{-1} yr^{-1} in filtered air. A positive interaction between ozone and nitrogen was found for the medium sized root starch concentrations. For all other compartments no interaction could be detected.

Table 3: Effect of ozone fumigation (O_3) and nitrogen fertilisation (N) on soluble carbohydrate concentrations of fine roots and starch concentrations of needles, stems, and roots. Data were corrected for soil pH. Values are means ± SE; n = 256 observations in 4 groups; mixed effects linear model: (*) = $p < 0.1$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$; ns = not significant.

treatment	**starch** [mg g_{dw}^{-1}]			**soluble carbohydrates in fine roots** [µg g_{dw}^{-1}]			
	needles	**stems**	**roots**	**alcohols**	**mono-saccharides**	**di- & tri-saccharides**	**total**
filtered							
0 [kg N ha^{-1} yr^{-1}]	9.44 ± 1.73	8.58 ± 0.88	68.66 ± 2.46	7.88 ± 0.34	9.39 ± 0.31	33.04 ± 1.23	50.31 ± 1.48
20 [kg N ha^{-1} yr^{-1}]	8.76 ± 1.54	7.14 ± 0.66	66.48 ± 3.35	8.16 ± 0.32	9.13 ± 0.29	30.21 ± 1.26	47.49 ± 1.40
40 [kg N ha^{-1} yr^{-1}]	7.58 ± 1.67	5.46 ± 1.02	65.41 ± 3.21	6.65 ± 0.38	9.42 ± 0.30	32.32 ± 1.17	48.38 ± 1.20
80 [kg N ha^{-1} yr^{-1}]	12.62 ± 2.72	11.48 ± 1.74	74.35 ±2.92	7.23 ± 0.39	10.13 ± 0.33	34.64 ± 1.06	52.00 ± 1.18
ambient							
0 [kg N ha^{-1} yr^{-1}]	3.90 ± 0.39	4.22 ± 0.30	52.21 ± 2.80	8.98 ± 0.39	10.71 ± 0.31	30.95 ± 1.15	50.63 ± 1.34
20 [kg N ha^{-1} yr^{-1}]	5.94 ± 0.88	6.63 ± 0.62	56.97 ± 3.11	7.34 ± 0.30	10.38 ± 0.34	34.42 ± 0.97	52.13 ± 1.14
40 [kg N ha^{-1} yr^{-1}]	5.15 ± 0.68	6.61 ± 0.64	63.18 ± 2.99	6.95 ± 0.24	9.92 ± 0.34	32.00 ± 1.06	48.86 ± 1.39
80 [kg N ha^{-1} yr^{-1}]	2.74 ± 0.23	5.10 ± 0.37	67.76 ± 2.56	6.81 ± 0.26	12.06 ± 0.43	35.86 ± 1.18	54.73 ± 1.38
O3	***	***	***	ns	***	ns	*
N	***	(*)	(*)	***	***	**	*
O3*N	ns	ns	*	ns	ns	ns	ns

3.4.5 Soluble carbohydrates

Results of soluble carbohydrate concentrations of fine roots are given in Table 3. Sugar alcohol and di- and trisaccharide concentrations of the fine roots showed no reaction to the ozone fumigation. Monosaccharides were significantly enhanced by ozone as well as the total soluble carbohydrate concentrations. The latter enhancement was mainly caused by the increase of the monosaccharide concentrations. Nitrogen had a significant effect on all of the measured soluble carbohydrates. However, while alcohols were reduced, monosaccharides as well as di- and trisaccharides were enhanced by nitrogen fertilisation. No interaction of nitrogen fertilisation and ozone fumigation for any soluble carbohydrate concentrations of fine roots was found.

3.4.6 Nutrient status

The results of the nutrient analysis of the needles are given in Table 4. For the investigated nutrients, no interactions between nitrogen fertilisation and ozone fumigation were found. Nitrogen fertilisation significantly increased the needle nitrogen concentrations, whereas ozone fumigation had no effect.

Table 4: Effect of ozone fumigation (O_3) and nitrogen fertilisation (N) on nutrient concentrations and nutrient ratios of spruce needles. Data were corrected for soil pH. Values are means ± SE; n = 96 observation in 4 groups; mixed effects linear model: (*) = $p < 0.1$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$; ns = not significant.

treatment	**nutrients** [mg g_{dw}^{-1}]						**nutrient ratios**			
	N	**P**	**K**	**Ca**	**Mg**	**Mn** [µg g_{dw}^{-1}]	**N : P**	**N : K**	**N : Ca**	**N : Mg**
filtered										
0 [kg N ha^{-1} yr^{-1}]	19.31 ± 0.68	2.54 ± 0.12	5.47 ± 0.40	9.64 ± 0.80	1.50 ± 0.07	96.7 ± 9.90	7.83 ± 0.52	3.68 ± 0.27	2.15 ± 0.17	13.23 ± 1.00
20 [kg N ha^{-1} yr^{-1}]	19.08 ± 0.85	2.52 ± 0.13	5.24 ± 0.31	9.88 ± 0.53	1.41 ± 0.08	104.33 ± 4.71	7.79 ± 0.42	3.69 ± 0.17	1.99 ± 0.12	14.25 ± 1.30
40 [kg N ha^{-1} yr^{-1}]	19.34 ± 0.54	2.27 ± 0.11	5.05 ± 0.23	10.06 ± 0.56	1.62 ± 0.08	107.3 ± 6.74	8.68 ± 0.35	3.89 ± 0.19	1.98 ± 0.09	12.22 ± 0.51
80 [kg N ha^{-1} yr^{-1}]	20.13 ± 0.79	2.11 ± 0.10	4.71 ± 0.28	9.84 ± 0.60	1.29 ± 0.08	72.92 ± 8.66	9.70 ± 0.39	4.39 ± 0.26	2.17 ± 0.15	16.17 ± 1.33
ambient										
0 [kg N ha^{-1} yr^{-1}]	18.04 ± 0.77	2.42 ± 0.13	4.79 ± 0.31	10.42 ± 0.47	1.52 ± 0.08	120.88 ± 14.46	7.53 ± 0.34	3.99 ± 0.27	1.77 ± 0.08	12.03 ± 0.51
20 [kg N ha^{-1} yr^{-1}]	18.65 ± 0.56	2.59 ± 0.09	4.84 ± 0.31	10.83 ± 0.98	1.55 ± 0.06	106.40 ± 7.71	7.32 ± 0.35	4.12 ± 0.29	1.83 ± 0.13	12.21 ± 0.51
40 [kg N ha^{-1} yr^{-1}]	18.77 ± 0.75	2.31 ± 0.17	4.39 ± 0.34	9.64 ± 0.54	1.45 ± 0.09	67.29 ± 10.89	8.50 ± 0.54	4.49 ± 0.28	2.01 ± 0.12	13.55 ± 0.76
80 [kg N ha^{-1} yr^{-1}]	19.83 ± 0.60	1.97 ± 0.09	4.25 ± 0.32	10.81 ± 0.62	1.36 ± 0.08	95.42 ± 10.17	10.20 ± 0.33	5.02 ± 0.32	1.88 ± 0.09	15.25 ± 1.07
O_3	ns	ns	*	ns	ns	ns	ns	**	*	ns
N	*	***	**	ns	*	**	***	***	ns	***
O_3*N	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Phosphorus, potassium, magnesium, and manganese concentrations in needles were significantly reduced with nitrogen fertilisation. Ozone fumigation only affected the potassium concentrations negatively. No effect of either treatment was detected for the calcium concentrations of the needles.

The ratios of N : P, N : K, and N : Mg were significantly increased with increasing N fertilisation. No differences for N : Ca were found. Ozone had no effect on nutrient ratios except for N : K which was increased and N : Ca which was decreased by the fumigation.

3.5 Discussion

3.5.1 Discoloration

Ozone exposure can cause chlorotic mottle in conifer species, identified as chlorotic spots and yellowing of the needles, which was reported in several studies (Matyssek and Innes, 1999). These symptoms were also observed in our experiment, where the needle discoloration was significantly increased by ozone fumigation. On the other hand, nitrogen fertilisation also enhanced the needle yellowing, even though nitrogen fertilisation rather leads to an increase in chlorophyll and therefore to darker green needles (Utriainen and Holopainen, 2001a). The observed effects cannot be explained by nutrient imbalances either, as the nutrient concentrations and nutrient ratios were still within normal to optimal ranges (see below) (Stefan et al., 1997).

3.5.2 Shoot elongation

In our experiment, nitrogen had a significant positive impact on shoot growth of spruce saplings. These findings are consistent with various other publications, e.g. Kainulainen et al. (2000) or Utriainen and Holopainen (2001a), where additional nitrogen also enhanced shoot growth in *Picea abies*. Similar findings were published for other conifer species, e.g. *Pinus sylvestris* (Manninen et al., 2002; Utriainen et al., 2001; Utriainen and Holopainen, 2001b). Also our findings of a negative growth effect of ozone are in accordance with findings of other groups (Karlsson et al., 1996; Ottoson et al., 2003; Wallin et al., 2002). Nitrogen fertilisation and ozone fumigation did not interact, which means that the observed effects can be seen as additive, where nitrogen fertilisation might level off the negative impact of ozone on shoot growth. This is in accordance with Utriainen and Holopainen (2001a) or Kainulainen et al. (2000) who also found no interaction of ozone and nitrogen on growth of *Picea abies*, or *Pinus sylvestris*.

3.5.3 Biomass

Biomass production was significantly enhanced by nitrogen in aboveground as well as in belowground plant parts. Similar findings were presented for *Picea abies* seedlings (e.g. Lippert et al., 1996; Seith et al., 1996; Utriainen and Holopainen, 2001a) or *Pinus sylvestris* (e.g. Utriainen and Holopainen, 2001b). In these studies, the aboveground plant parts were stronger influenced than the roots, leading to an increase in the shoot : root ratio. This was not found in our experiment, where the ratio was unaffected by nitrogen fertilisation. Similar results were found for *Pinus taeda* by Tjoelker and Luxmoore (1991), where biomass was also enhanced, but not the ratio of aboveground to belowground biomass. Ozone, which affected neither shoot, nor root dry weight significantly, changed the ratio towards the belowground plant parts. To our knowledge, most studies show rather no effect (e.g. Landolt et al., 2000; Lippert et al., 1996; Utriainen and Holopainen, 2001b), or an increase (Ottoson et al., 2003; Wallin et al., 1996) in the shoot : root ratio of spruce fumigated with ozone, except for Landolt et al. (1999) and Rantanen et al. (1994), who also stated a slight decrease in spruce trees. Significant decreases were also found for *Pinus sylvestris* by Kainulainen et al. (2000) or for *Pinus taeda* by Mahoney et al. (1985) and Tjoelker and Luxmoore (1991).

Again, no interaction of ozone or nitrogen was detected in our experiment. Similarly, no interactions of the two pollutants were detected for other conifers (Kainulainen et al., 2000) or broad-leafed species (Pell et al., 1995), with the study of Pääkkönen and Holopainen (1995) as an exception.

3.5.4 Starch concentrations

The starch concentrations of the medium sized roots and stems were slightly increased by nitrogen, but needle starch concentrations were overall reduced, with the exception of the highest N treatment in filtered air. To our knowledge, only Wallenda et al. (1996) have investigated the effect of nitrogen fertilisation on the starch concentrations of spruce roots. Similar to our results, they found decreased needle starch concentrations, but root starch concentrations were decreased as well. This has also been shown for roots of *Pinus ponderosa* (Grulke et al., 2001) and *Fagus sylvatica* (Thomas et al., 2002).

Ozone is thought to restrict phloem loading und thus assimilate translocation from shoot to roots (Cooley and Manning, 1987; Skärby et al., 1998). This can result in carbon accumulation in aboveground plant parts and a reduction in the roots. Lux et al. (1997) and Braun et al. (2004) found decreased root and twig starch concentrations of *Picea abies* seedlings under enhanced ozone fumigation, but increases in needles. Similar results have been shown for mature Ponderosa pines with decreased root and needle starch concentrations, but an increase in bole starch concentrations (Grulke et al., 2001). In our study however, starch concentrations decreased not only in roots, but in all investigated plant parts. An overall decline in the starch concentrations could be explained, as photosynthesis is often impaired by ozone whereas respiration increases (Dizengremel, 2001). This may lead to an overall decrease in carbohydrate production. In addition, carbon metabolism is modified under ozone exposure for detoxification processes, protection and repair of the cell structure (Dizengremel, 2001). As the spruce saplings showed visible injuries from the second year on, it is likely that photosynthates were needed for repair mechanisms resulting in a negative impact on carbohydrate accumulation.

Interactions of ozone fumigation and nitrogen fertilisation were found for the root starch concentrations only. The interaction was antagonistic, which means that higher loads of nitrogen alleviated the negative effect of ozone. Thomas et al. (2002) found a similar impact on root starch concentrations of mature *Fagus sylvatica* trees. In their study, ozone and nitrogen reduced the starch concentrations significantly, but the combination of the two factors alleviated each other under high pollution levels.

3.5.5 Soluble carbohydrates

Except for the sugar alcohols, nitrogen had a positive effect on the root sugar concentrations. Similarly, Wallenda et al. (1996) found decreased sugar alcohol concentrations of mannitol, but also of trehalose. Mannitol and trehalose are the main fungus-specific soluble carbohydrates in *Picea abies* (Wallenda et al., 1994). In our experiment mannitol was significantly reduced and trehalose also showed a negative trend with nitrogen fertilisation ($p_{mannitol} < 0.001$, $p_{trehalose} = 0.079$; data not shown), indicating a possible change in mycorrhizal infestation, or mycorrhizal vitality of the spruce roots. Monosaccharides, di- and trisaccharides, and total soluble carbohydrates all increased in the fine roots under enhanced nitrogen fertilisation. This is in contrast with studies of Grulke et al. (2001) who found a significant decrease of monosaccharides in the roots of Ponderosa pines under enhanced nitrogen deposition.

Also ozone fumigation increased the monosaccharide and the total soluble carbohydrate concentrations in spruce roots, whereas sugar alcohols and the di-and trisaccharides were not affected. As mentioned above, ozone might impair phloem loading and therefore alter carbon partitioning, causing a decrease in root carbohydrate concentration and an accumulation of carbohydrates in aboveground plant parts. But, according to Mahoney et al. (1985), the relative strength of the root sink may modify the effect of ozone on partitioning. Their results suggested that the ectomycorrhizal fungus *Pisolithus tinctorius* was capable of significantly modifying root sink strength and increasing the root demand for photosynthate of loblolly pine seedlings. Rantanen et al. (1994) and also Reich et al. (1986) provided evidence, that mycorrhizal short-root formation of *Picea abies* and *Pinus strobus* seedlings was enhanced under ozone stress. It is therefore possible, that the ozone fumigation in our experiment influenced mycorrhizal infection of the roots, leading to a changed carbohydrate demand. But of the two investigated main fungus-specific soluble carbohydrates, trehalose was the only one to show a positive effect of ozone fumigation (data not shown, $p_{trehalose} = 0.053$, $p_{mannitol} = 0.435$). An increase in soluble sugar concentrations from breakdown of stored reserves, such as starch is also possible (Andersen, 2003).

The interaction term of ozone fumigation and nitrogen fertilisation was not significant for any of soluble carbohydrate concentrations. As both pollutants increased the monosaccharide and the total soluble carbohydrate concentrations, an additive effect of nitrogen fertilisation and ozone fumigation can be assumed.

3.5.6 Nutrients

Even though nitrogen had a significant effect on the nutrient concentrations and nutrient ratios in spruce needles, all measured values were still in normal to optimal ranges (Stefan et al., 1997). The observed changes however, indicate, that a longer lasting nitrogen fertilisation might result in further decreases of nutrients and unbalanced nutrient ratios as has been shown by Flückiger and Braun (1998). Increased nitrogen concentrations of needles make spruce trees more susceptible towards sucking insects and pathogens (Flückiger and Braun, 1998; Roelofs et al., 1985). In addition, P concentrations are likely to decrease further, as an enhanced nitrogen supply is known to increase the P demand of plants (Phoenix et al., 2003) and lead to a P deficiency in trees (Mohren et al., 1986). Ozone had only an effect on potassium concentrations which were significantly decreased and, in consequence, the ratio of N : K was increased. For *Pinus sylvestris*, changes in the potassium concentrations of needles under ozone fumigation are reported by Utriainen and Holopainen (2001b). In their study, however, the potassium concentrations were increased. They also found changes in the Ca concentrations, whereas in our study only the ratio of N : Ca was changed under ozone fumigation. No interaction of ozone and nitrogen on nutrient concentrations were found. This has also been reported by Utriainen and Holopainen (2001b).

3.6 Conclusion

Both pollutants showed significant effects on spruce saplings. Ozone affected the plants in a more negative way, resulting in visible damages, reduced growth, and decreased starch concentrations in different plant parts. Nitrogen on the other hand had a more positive effect, enhancing plant growth, biomass production, starch concentrations, and soluble carbohydrate concentrations. Changes in the fungus-specific sugar alcohols mannitol and trehalose might be signs of alterations in the mycorrhization under nitrogen fertilisation and ozone fumigation. An interaction of the two pollutants was only found for the root starch concentrations, where nitrogen and ozone had an antagonistic effect. All other investigated parameters showed no interacting effects of ozone fumigation and nitrogen fertilisation.

3.7 Acknowledgements

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4. EFFECTS OF SIMULTANEOUS OZONE EXPOSURE AND NITROGEN LOADS ON CARBOHYDRATE CONCENTRATIONS, BIOMASS, GROWTH, AND NUTRIENTS OF YOUNG BEECH TREES (*FAGUS SYLVATICA* L.)

4.1 Abstract

Beech seedlings were grown under different nitrogen fertilisation regimes (0, 20, 40, 80 kg N ha^{-1} yr^{-1}) for three years and were fumigated with either charcoal-filtered (F) or ambient air (O_3). Nitrogen fertilisation increased leaf necroses, aphid infestations, and nutrient ratios in the leaves (N : P and N : K), as a result of decreased phosphorus and potassium concentrations. For plant growth, biomass accumulation, and starch concentrations a positive nitrogen effect was found, but only for fertilisations of up to 40 kg N ha^{-1} yr^{-1}. The highest nitrogen load, however, reduced leaf area, leaf water content, growth, biomass accumulation, and starch concentrations, whereas soluble carbohydrate concentrations were enhanced. The ozone fumigation resulted in reduced leaf area, leaf water content, shoot growth, root biomass accumulation, and decreased starch, phosphorus, and potassium concentrations, increasing the N : P and N : K ratios. A combinatory effect of the two pollutants was detected for the leaf area and the shoot elongation, where ozone fumigation amplified the nitrogen effects.

4.2 Introduction

Nitrogen has previously been a limiting factor in forest growth, but N depositions have increased during the second half of the last century to such an extent, that the critical loads for forests of 10 – 20 kg N ha^{-1} yr^{-1} (Achermann and Bobbink, 2003) are exceeded in about 90 % of Swiss forest by now (Rihm, 1996). Ambient ozone doses have also increased in the last decades, often exceeding the critical level of 5 ppm h AOT 40 daylight (accumulated exposure over a threshold of 40 ppb) (Karlsson et al., 2004). Both pollutants are known to affect growth and biomass. In addition impacts on storage carbohydrate concentrations have been suggested for ozone as well as nitrogen. Despite the broad knowledge on ozone and nitrogen impacts alone, the combinatory effects of these two pollutants are poorly understood.

In a recently published paper, we examined the simultaneous exposure of young spruce trees (*Picea abies*) towards ozone and nitrogen fertilisation (Thomas et al., 2005). Ozone proofed to have negative impacts on growth and also starch concentrations in different plant parts. Nitrogen on the other hand, with fertilisation rates of up to 80 kg N ha^{-1} yr^{-1} increased growth, biomass accumulation, soluble carbohydrate concentrations and also enhanced starch concentrations slightly. Except for root starch concentrations, where an antagonistic interaction was observed, the two pollutants showed no interactive effects.

In this parallel study, we examined young beech trees under the same ozone and nitrogen treatments. Broad-leafed tree species are supposed to be more sensitive to ozone compared to evergreen tree species. According to Pell et al. (1995) an increased nitrogen supply can render *Populus tremuloides* seedlings less sensitive towards ozone concerning biomass accumulation. This was also shown for *Betula pendula* seedlings, where no negative ozone effects were observed at sufficiently high nitrogen levels (up to 150 kg N ha^{-1} yr^{-1}) (Pääkkönen and Holopainen, 1995). Also Thomas et al. (2002) have shown in a gradient study with mature *Fagus sylvatica*, that both ozone and nitrogen exposure were correlated with reduced starch concentrations in roots, but at high exposure levels the two pollution factors alleviated the effect of each other.

European beech is one of the major tree species in Switzerland and rather sensitive to ozone (Karlsson et al., 2004). In our three year-study we examined the combinatory effects of ozone fumigation and nitrogen fertilisation on growth, biomass production, carbohydrate concentrations, and nutrient status of beech. The aim of our experiment was to test for interactive effects of nitrogen fertilisation and ozone fumigation and for possible response differences between spruce and beech seedlings under exposure to both pollutants.

4.3 Materials and Methods

4.3.1 Plants

In March 2000, European beech seedlings (*Fagus sylvatica*) from a local provenance (altitude 400 m asl) were planted together with spruce saplings into the bare soil within eight units (see below). The soil was a calcium-rich Luvisol, with former agricultural use and pH ($CaCl_2$) values between 5.2 and 7.1. The plants were watered with tap water in conditions of dry weather. Results of the spruce trees are not mentioned here, but are published in another paper (Thomas et al., 2005).

4.3.2 Fumigation system

The fumigation system used is described in detail by Thomas et al. (2005). Eight chamberless fumigation units were constructed according to Leyendecker et al. (1996) at the research site in Schönenbuch (latitude 47.5 °, altitude 400 m asl), Switzerland. Four units were fumigated with charcoal filtered air (C1, Gertsch Filter, Balsthal, Switzerland) and four with ambient air, which was enriched with ozone to compensate for the system loss. The ozone data from April to September of each experimental year are given in Table 1. Average concentrations and AOT40 (Fuhrer 1997) were calculated during daylight hours. A cumulative stomatal uptake for European beech was computed according to Emberson et al. (2000b) and Karlsson et al. (2004) using either no threshold or a threshold of 1.6 nmol m^{-2} s^{-1}.

Table 1: Ozone data during the 3 year experimental period between April and September (average of the four fumigation units ± standard deviation).

		Mean daylight hours [ppb]	AOT40 daylight hours [ppm h]	Cumulative O_3 uptake threshold zero [mmol m^{-2} proj. area]	Cumulative O_3 uptake threshold 1.6 nmol m^{-2} s^{-1} [mmol m^{-2} proj. area]
2000	filtered	19.4 ± 0.6	0.1 ± 0.0	2593 ± 101	0.4 ± 0.1
2000	ambient	37.6 ± 3.1	11.5 ± 4.6	4871 ± 426	5.9 ± 1.4
2001	filtered	22.1 ± 2.2	0.5 ± 0.4	2084 ± 186	0.4 ± 0.1
2001	ambient	35.2 ± 5.7	7.2 ± 5.3	3177 ± 555	2.0 ± 1.3
2002	filtered	28.3 ± 3.5	2.7 ± 2.5	3945 ± 427	3.4 ± 1.3
2002	ambient	47.4 ± 0.2	22.1 ± 0.1	6458 ± 90	11.5 ± 0.3

4.3.3 Nitrogen treatment

Each fumigation unit was divided into quarters for different nitrogen fertilisation. Plastic foil was used to separate the quarters and the whole units from the surrounding soil to a depth of 70 cm. The quarters received liquid nitrogen fertilisation (NH_4NO_3) of 0, 20, 40, and 80 kg ha^{-1} yr^{-1} in six monthly treatments during the growing seasons (April – September) in 2000, 2001, and 2002. Modelled background N deposition was 15 kg N ha^{-1} yr^{-1}. Seventy percent of this N input is in gaseous form (NH_3, NO_2, and HNO_3), which may have been reduced by the filtration.

4.3.4 Measurements in the field

Annual shoot elongation was measured each year after the vegetation period. Drought-like edge necroses of leaves were first observed after short drought periods in summer 2001 and also during the vegetation period of 2002. In July 2001, 2002, and September 2002 they were evaluated using ten different categories from no to strong injuries (0 - 9). Infestations with *Phyllaphis fagi* were also observed in 2001 and 2002 and were evaluated in July 2002 (ten categories from 0 - 9).

4.3.5 Collection of Plant material

4.3.5.1 Leaves

In summer 2002 leaves without necroses were collected for leaf area and water content measurements. After scanning for leaf area (WinFOLIA, Régent Instruments Inc., Québec, Canada) and determination of the fresh weight (fw), the samples were dried at 60 °C for two days, weighed (dw) and milled for nutrient analysis. Water content was calculated as wc = (fw – dw) * dw^{-1}. Three leaf samples of each fertilisation treatment of each unit were digested with H_2SO_4/H_2O_2 and a Se/$LiSO_4$ catalyst, following Allen (1989). Nitrogen and phosphorus were measured colorimetrically (Walinga et al., 1995), whereas Ca, K, and Mg were analysed with an AAS (SpectrAA-10, Varian, IL, USA). Apple leaves (National Institute of Standards and Technology, Gaithersburg, MD, USA) were used as a standard.

4.3.5.2 Roots and Stems

After the third growth period (winter 2002/03), all trees were harvested destructively for biomass determination, soluble carbohydrate analysis of fine roots (diameter: ≤ 0.5 mm), starch analysis of medium sized roots (diameter: > 1.0 to ≤ 2.5 mm), and current shoots. Roots were cleaned from adhering soil, separated in fractions, shock frozen in liquid nitrogen, lyophilised, and ball-milled, as described in detail by Thomas et al. (2002). Aboveground plant parts were washed carefully with cold water. Current shoots were separated, rinsed with deionised water and treated like the root samples. The remaining above- and belowground plant material was oven dried for at least 2 days at 70 °C and weighed for biomass determination.

4.3.6 Carbohydrate analysis

4.3.6.1 Soluble carbohydrates

Soluble carbohydrate analysis of the fine roots was performed after the method of Peuke et al. (2002) with an ion chromatograph (Dionex Corporation, Sunnyvale, CA, USA). The duration of a run lasted 80 minutes with a gradient of NaOH from 16 mM to 200 mM, using a CarboPac PA10 column plus guard column and an ED 50 electrochemical detector (Dionex Corporation, Sunnyvale, CA, USA). The following carbohydrates were detected: sugar alcohols: arabitol, mannitol, myo-inositol, and sorbitol; monosaccharides: arabinose, fucose, fructose, galactose, glucose, mannoheptulose, mannose, and rhamnose; di- and trisaccharides: maltose, raffinose, sucrose, and trehalose. Xylose and mannose could not be separated and the peak was treated as mannose only, which occurs in higher concentrations than xylose in beech (Lux et al., 1997).

4.3.6.2 Starch

The starch concentrations of the roots and twigs were measured chromatographically as glucose units after the method of Thomas et al. (2005). The samples were mixed with insoluble polyvinylpyrrolidone (Polyclar AT, Serva, Heidelberg, Germany), prewashed with water to remove soluble carbohydrates and extracted with citrate-buffer solution and amyloglucosidase at 60 °C. After centrifugation, the samples were filtrated and kept frozen until injection into the same chromatographic system as used for the soluble carbohydrates.

Depending on the starch concentrations different dilutions were used for the different plant parts (roots: 100 x, stems: 50 x). Starch amounts were estimated by multiplying the concentrations with the above- and belowground biomass.

4.3.7 Statistics

For statistical analysis the software packages SYSTAT 10 (Systat Inc., Evanston, IL, USA) and S-PLUS 2000 (MathSOFT INC., Cambridge, MA, USA) were used. Shoot length, starch, and soluble carbohydrate concentrations were root-transformed. Sugars were combined to groups and tested as such (alcohols, monosaccharides, di- and trisaccharides, and total). In order to reduce the effects of gradients in soil conditions and light within the experimental field, blocks were formed which consisted of one filtered and one ambient air unit each (Schuster and von Lochow, 1991) and served as a random grouping variable in the statistical analysis (S-PLUS, linear mixed effects model). A confounding pH gradient remained nevertheless which necessitated its consideration as a covariate. The dependent variables were tested simultaneously against ozone and nitrogen including an interaction term. When no significant interaction of the two pollutants could be detected, the interaction term was excluded from the calculations. To show statistical significant differences between single treatments in the figures, a least-significant-difference test (SYSTAT) was used. The residuals of the tests were checked for normal distribution and the presence of outliers.

4.4 Results

4.4.1 Visible symptoms and aphid infestations

Nitrogen fertilisation above 20 kg N ha^{-1} yr^{-1} had an enhancing effect on the formation of leaf edge necrosis in all three evaluations ($p < 0.001$ each). Only in the last evaluation, in September 2002, also nitrogen fertilisation of 20 kg N ha^{-1} yr^{-1} increased the leaf damages significantly ($p = 0.006$) (Figure 1). Aphid infestation of the beech saplings was also positively related with nitrogen loads of 20, 40, and 80 kg N ha^{-1} yr^{-1} ($p_{20} = 0.037$, $p_{40} < 0.001$, $p_{80} < 0.001$), whereas ozone fumigation had no significant effect (Figure 2). An interaction of the two pollutants was not detected.

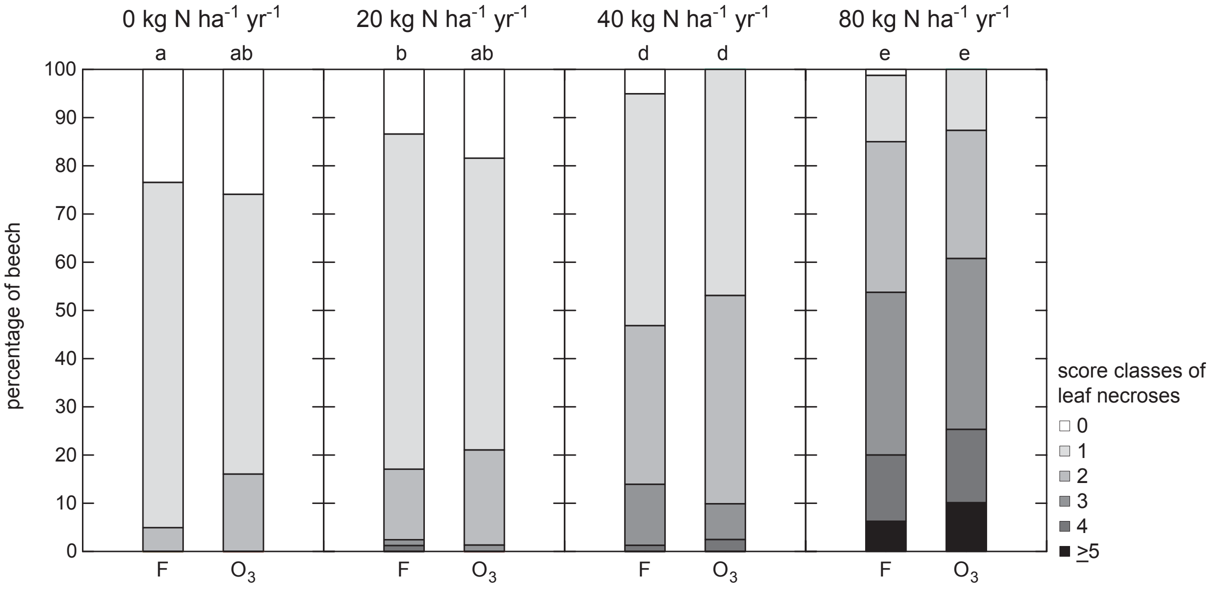

Figure 1: Percentage of beech seedlings showing leaf necroses with different nitrogen treatments in filtered (F) or ambient air (O_3) in September 2002. Differences ($p < 0.05$) are indicated by different letters.

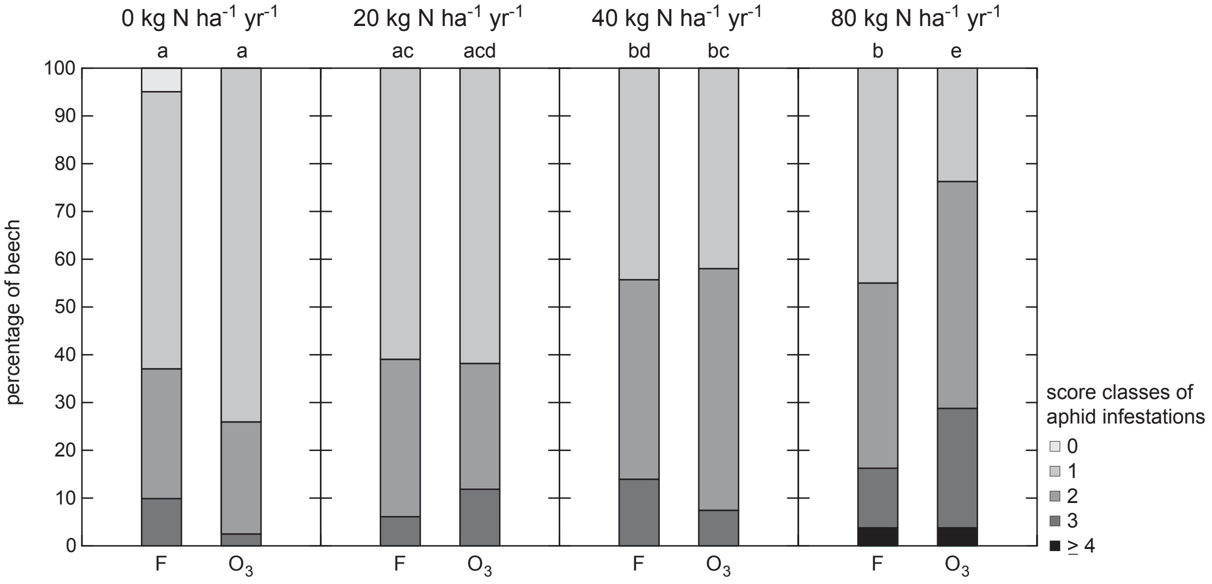

Figure 2: Percentage of beech seedlings showing infestations with *Phyllaphis fagi* under different nitrogen fertilisation treatments and in filtered (F) or ambient air (O_3). Differences ($p < 0.05$) are indicated by different letters.

4.4.2 Leaf area and water content

Results of the leaf parameter measurements are given in Table 2 and 3. Ozone fumigation led to smaller leaves with a lower specific leaf area, as was shown by lower fresh weight (fw), dry weight (dw), leaf area (LA), and LA / dw ratio. Also the leaf water content (lwc) was significantly reduced under ozone fumigation. Nitrogen fertilisation of 20 and 80 kg N ha^{-1} yr^{-1} resulted in smaller leaves, mainly in seedlings grown in filtered air, as was indicated by the significant interaction term at these levels. For the specific leaf area (LA : dw) no combinatory effect could be found, but ozone alone and nitrogen fertilisations of 20 and 80 kg N ha^{-1} yr^{-1} reduced the LA : dw significantly.

A significant interaction was shown for fw and dw in plants receiving 20 kg N ha^{-1} yr^{-1}, leading to higher values in ozone fumigated beech seedlings. For the lwc no interaction effect was evident, but 40 kg N ha^{-1} yr^{-1} showed a positive and 80 kg N ha^{-1} yr^{-1} a negative effect on the lwc in all plants.

Table 2: Leaf parameters for the different treatments. Given are leaf area (LA), fresh weight (fw), dry weight (dw), relative water content (RWC), and specific leaf area (LA : dw). Values are means ± SE; n = 288 observations in 4 groups.

	LA	**fw**	**dw**	**RWC**	**LA : dw**
filtered	[cm^2]	[mg]	[mg]	[%]	[cm^2 mg^{-1}]
0 kg N ha^{-1} yr^{-1}	16.44 ± 0.72	178.13 ± 9.35	87.07 ± 4.67	105.36 ± 1.45	0.193 ± 0.002
20 kg N ha^{-1} yr^{-1}	15.12 ± 0.54	168.91 ± 6.78	82.44 ± 3.41	105.34 ± 1.58	0.166 ± 0.005
40 kg N ha^{-1} yr^{-1}	15.63 ± 0.65	171.60 ± 8.59	81.67 ± 4.17	110.51 ± 1.85	0.196 ± 0.005
80 kg N ha^{-1} yr^{-1}	14.88 ± 0.56	169.07 ± 8.06	84.53 ± 4.02	99.98 ± 1.00	0.180 ± 0.004
ambient					
0 kg N ha^{-1} yr^{-1}	14.25 ± 0.59	154.07 ± 8.60	76.98 ± 4.30	99.84 ± 1.61	0.190 ± 0.004
20 kg N ha^{-1} yr^{-1}	15.58 ± 0.57	174.01 ± 7.79	85.49 ± 3.71	103.13 ± 2.02	0.185 ± 0.003
40 kg N ha^{-1} yr^{-1}	15.02 ± 0.53	165.36 ± 7.17	81.97 ± 3.36	102.14 ± 1.72	0.187 ± 0.004
80 kg N ha^{-1} yr^{-1}	14.71 ± 0.76	164.75 ± 9.51	84.65 ± 4.78	94.22 ± 2.02	0.176 ± 0.004

Table 3: Statistical analysis of the treatment effects of ozone fumigation (O_3) and nitrogen fertilisation (Nx) on shoot elongation, biomass accumulation, and leaf parameters according to the mixed effects linear model. The interaction term was only considered, where it was significant. Increasing or decreasing effects are indicated as „+“ or „-“ with indication of the significance level as (+), (-): $p < 0.1$; +, -: $p < 0.05$; + +, - -: $p < 0.01$; + + +, - - - : $p < 0.001$; n.s.: not significant. Data were corrected for soil pH. Observations in 4 groups, n = 256 - 288.

		O_3	**N20**	**N40**	**N80**
Elongation					
2002 + 2001	main effects	n.s.	n.s.	+	n.s.
	O_3 × N		+	n.s.	-
Biomass					
aboveground	main effects	n.s.	+	++	- -
belowground	main effects	(-)	n.s.	n.s.	- -
above : below	main effects	n.s.	(+)	n.s.	+
Leaves					
water content	main effects	- - -	n.s.	+	- - -
fresh weight	main effects	-	n.s.	n.s.	n.s.
	O_3 × N		+	n.s.	n.s.
dry weight (dw)	main effects	-	n.s.	n.s.	n.s.
	O_3 × N		+	n.s.	(+)
leaf area (LA)	main effects	- -	(-)	n.s.	-
	O_3 × N		+	n.s.	+
LA / dw	main effects	-	-	n.s.	- - -

4.4.3 Elongation and biomass

Annual elongation of 2001 and 2002 were combined to total shoot length at final harvest (Figure 3 and Table 2). Nitrogen fertilisation of 20 and 40 kg N ha^{-1} yr^{-1} increased shoot elongation, whereas at 80 kg N ha^{-1} yr^{-1} growth was suppressed. The observed combinatory effect revealed that ozone fumigation intensified the impact of the nitrogen fertilisation at 20 and 80 kg N ha^{-1} yr^{-1}. Saplings fumigated with ozone showed a stronger growth enhancement at 20 and also a stronger growth suppression at 80 kg N ha^{-1} yr^{-1} compared to plants grown in filtered air.

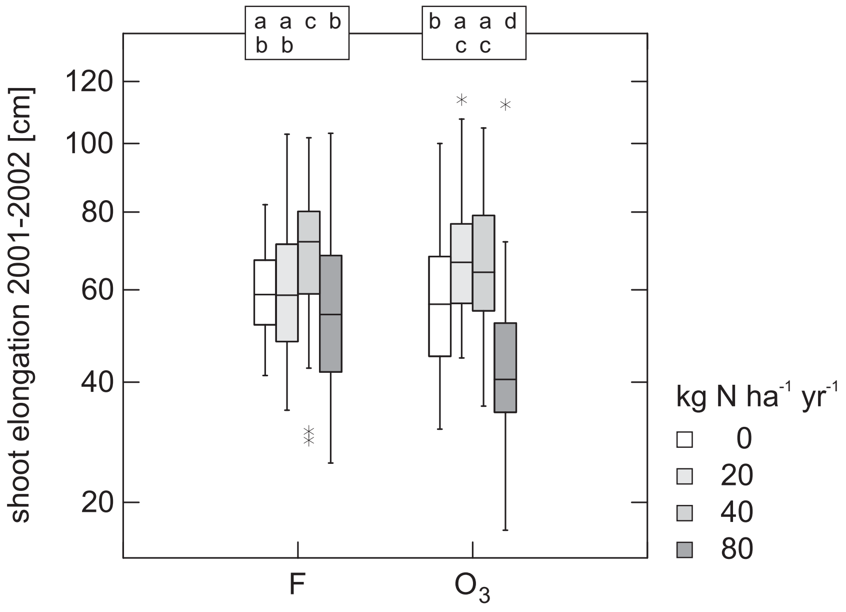

Figure 3: Effects of ozone fumigation and different nitrogen fertilisation treatments on shoot elongations of beech seedlings of two consecutive years (2001 and 2002). F: filtered air, O_3: ambient air. Differences ($p < 0.05$) are indicated by different letters, y-axes are square-root-transformed. Statistics are given in Table 3.

Total aboveground biomass revealed similar impacts of nitrogen fertilisation, resulting in an increase at 20 and 40 kg N ha^{-1} yr^{-1} and a decrease at 80 kg N ha^{-1} yr^{-1} (Figure 4 and Table 2). Ozone or the combination of the two pollutants did not influence the aboveground biomass. For the belowground biomass, a negative impact of the highest nitrogen fertilisation was found, and also ozone fumigation slightly decreased root biomass ($p_{O3} = 0.065$), but no combinatory effect could be stated.

The differences in the ratio of aboveground : belowground biomass were very small, but showed an enhancement of shoot biomass by nitrogen fertilisation of 20 and 80 kg N ha^{-1} yr^{-1} (Table 2). Ozone and the combination of the two pollution factors had no effect.

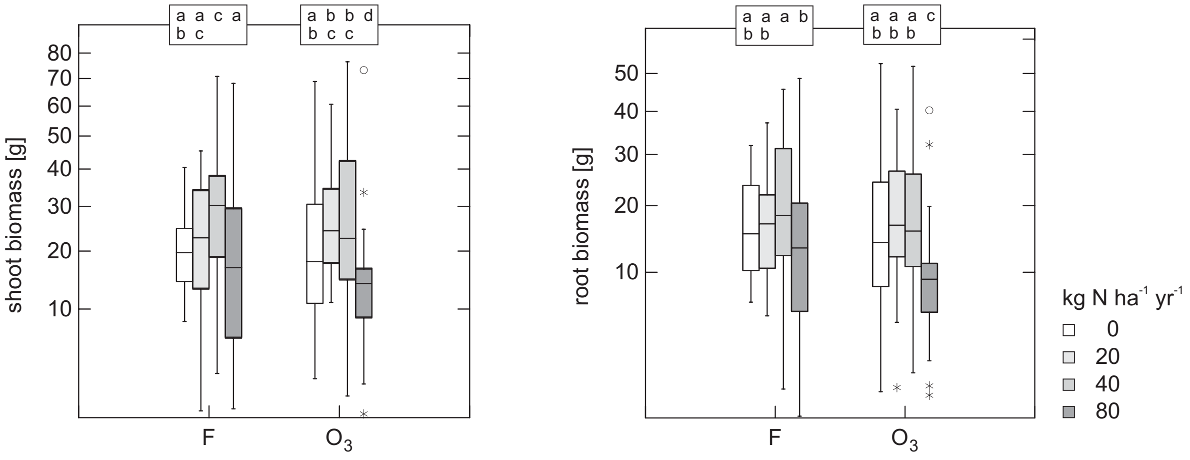

Figure 4: Effects of ozone fumigation and nitrogen fertilisation treatments on above- (left) and belowground (right) biomass accumulation in beech seedlings. F: filtered air, O_3: ambient air. Differences ($p < 0.05$) are indicated by different letters; y-axes are square-root-transformed. Statistics are given in Table 3.

4.4.4 Soluble carbohydrates

The results of the soluble carbohydrate analysis are given in Figure 5 and Table 4. The concentrations of monosaccharides, disaccharides, and total soluble carbohydrates in the fine roots were significantly increased by 80 kg N ha^{-1} yr^{-1}. There was no overall N effect on sugar alcohols but a significant interaction could be detected at 80 kg N ha^{-1} yr^{-1}. Nitrogen fertilisation at this level increased the sugar alcohol concentration significantly, but only in the plants grown in filtered air. In ozone fumigated plants, the changes in sugar alcohol concentrations were not significant. Other nitrogen treatments did not result in changes of the investigated carbohydrate groups. Ozone fumigation resulted in a significant increase of the fine root monosaccharide concentrations, but no other carbohydrate groups were affected. Also no interaction of the two pollutants could be detected for any of the investigated soluble carbohydrate groups, except for the sugar alcohols mentioned above.

Table 4: Statistical analysis of the treatment effects of ozone fumigation (O_3) and nitrogen fertilisation (Nx) on carbohydrate concentrations according to the mixed effects linear model. The interaction term was only considered, where it was significant. Increasing or decreasing effects are indicated as „+" or „-" with indication of the significance level as (+), (-): $p < 0.1$; +, -: $p < 0.05$; + +, - -: $p < 0.01$; + + +, - - - : $p < 0.001$; n.s.: not significant. Data were corrected for soil pH. Observations in 4 groups, n = 256.

		O_3	**N20**	**N40**	**N80**
Starch concentrations					
coarse roots	main effects	n.s.	n.s.	n.s.	-
	O_3 × N		n.s.	n.s.	(-)
stems	main effects	- -	n.s.	n.s.	- -
Starch amounts					
belowground	main effects	-	n.s.	+	- - -
aboveground	main effects	- -	+	+	-
	O_3 × N		(+)	n.s.	n.s.
Soluble carbohydrates					
sugar alcohols	main effects	n.s.	n.s.	n.s.	+
	O_3 × N		n.s.	n.s.	- -
monosaccharides	main effects	+ +	n.s.	n.s.	+ +
di- & trisaccharides	main effects	n.s.	n.s.	n.s.	+ +
total soluble carbohydrates	main effects	n.s.	n.s.	n.s.	+ +

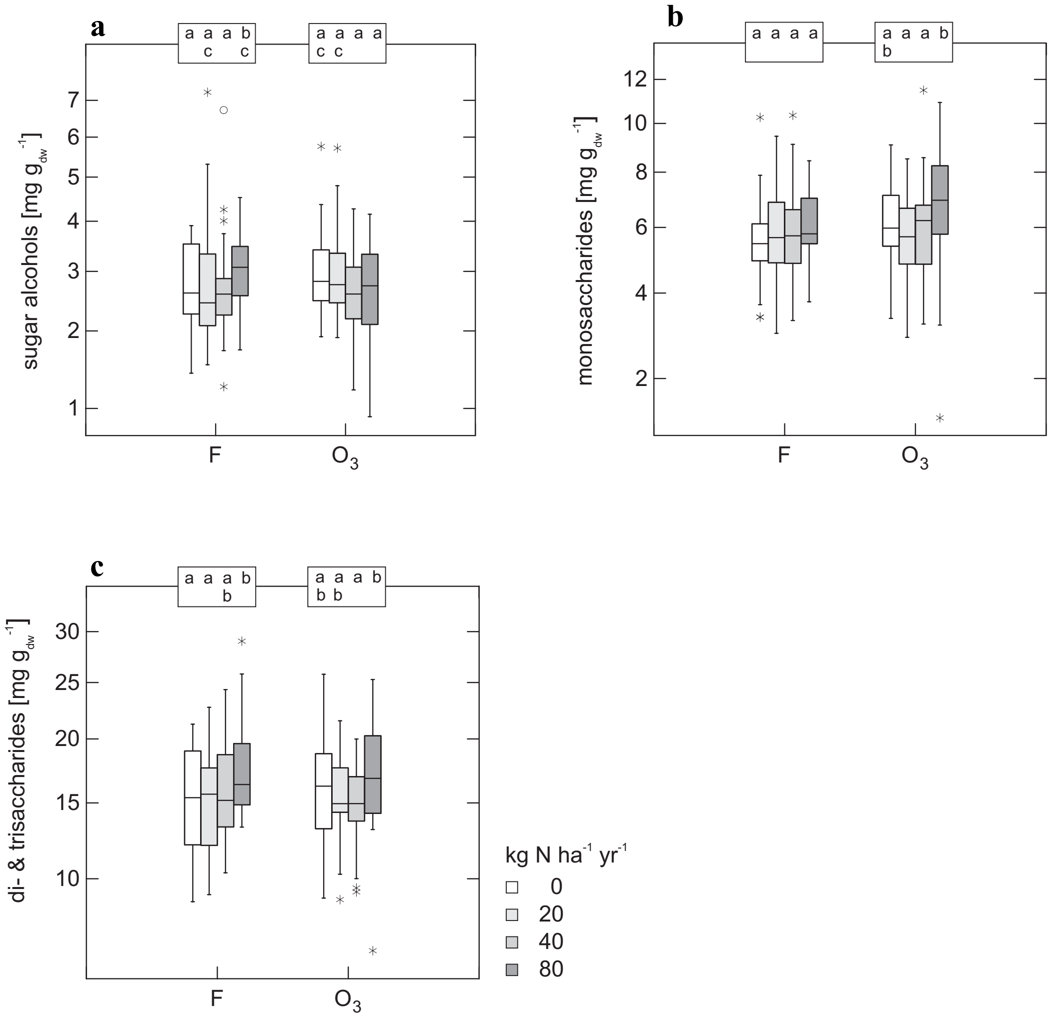

Figure 5: Effects of ozone fumigation and nitrogen fertilisation treatments on soluble carbohydrate concentrations in fine roots of beech seedlings. Given are sugar alcohol (a), monosaccharide (b), and di- and trisaccharide (c) concentrations. F: filtered air, O_3: ambient air. Differences ($p < 0.05$) are indicated by different letters; y-axes are square-root-transformed. Statistics are given in Table 4.

4.4.5 Starch concentrations

The results of the starch analysis are given in Figure 6 and Table 4. Root starch concentrations showed a slight interaction of ozone fumigation and nitrogen fertilisation at 80 kg N ha^{-1} yr^{-1} ($p = 0.060$). A decrease of the root starch concentration at this nitrogen level was found, which was more distinct in ozone-fumigated plants. For the stem starch concentrations no interaction was evident, but ozone fumigation and also nitrogen fertilisation of 80 kg N ha^{-1} yr^{-1} resulted in a significant decrease.

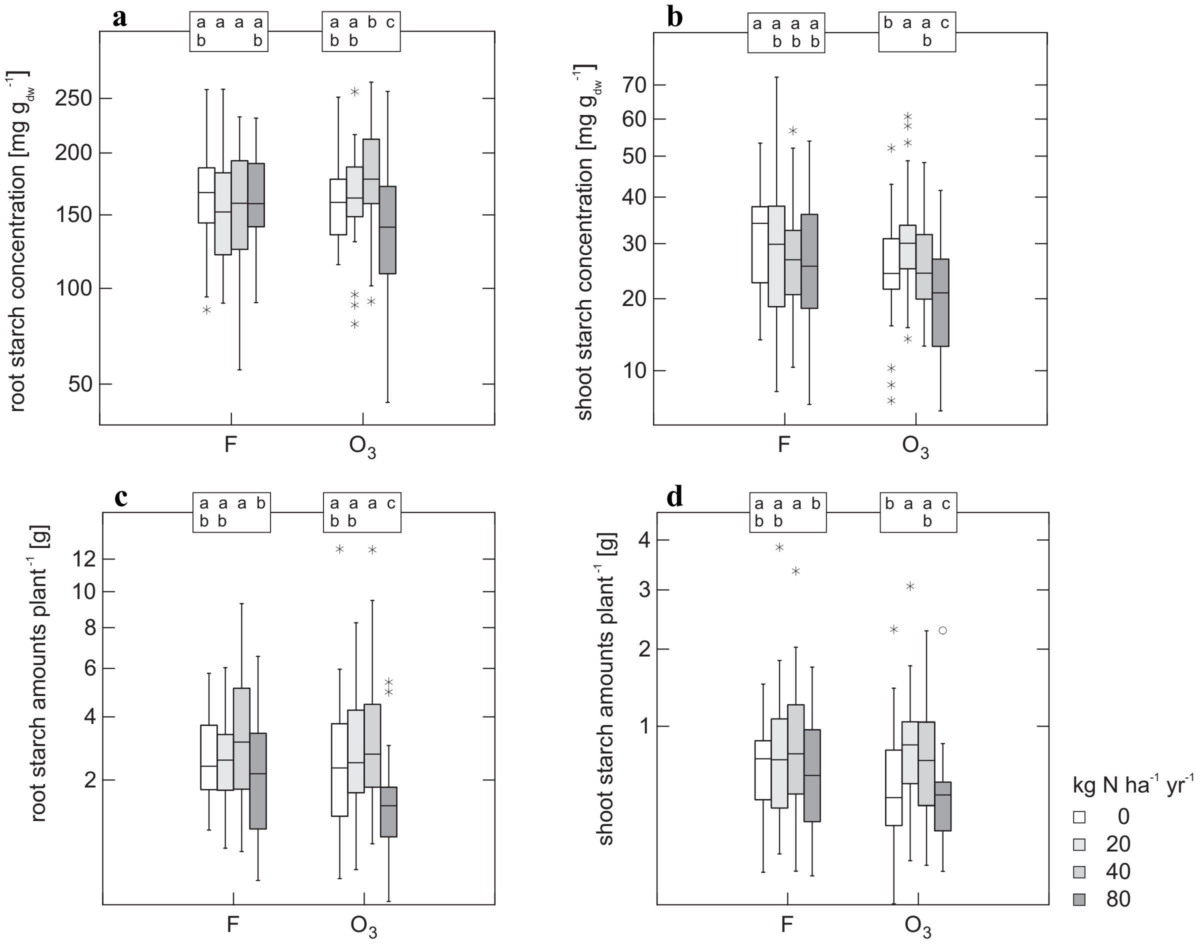

Figure 6: Effects of ozone fumigation and different nitrogen fertilisation treatments on starch concentrations and starch amounts in different plant parts of beech seedlings. Given are starch concentrations in coarse roots (a) and current year stems (b) and estimated starch amounts in below- (c) and aboveground (d) plant parts. F: filtered air, O_3: ambient air. Differences ($p < 0.05$) are indicated by different letters, y-axes are square-root-transformed. Statistics are given in Table 4.

To test for accumulation effects, the starch concentrations were extrapolated to total amounts in above- and belowground plant parts (Table 4). This calculation revealed a negative ozone impact on total starch concentrations in aboveground as well as in belowground plant parts with no combinatory effect evident. Nitrogen fertilisation increased total aboveground starch amounts at 20 and 40 kg N ha^{-1} yr^{-1}, whereas at 80 kg N ha^{-1} yr^{-1} a decrease was found. For the total root starch amounts similar results could be stated with a positive effect at 40 and a negative one at 80 kg N ha^{-1} yr^{-1}, whereas 20 kg N ha^{-1} yr^{-1} had no significant influence (Figure 6).

4.4.6 Nutrient status

4.4.6.1 Nutrient concentrations

The nutrient concentrations are given in Figure 7 and the corresponding statistics in Table 5. Nitrogen fertilisation or ozone fumigation showed no effect on the nitrogen or calcium concentrations in the leaves. Phosphorus concentrations were significantly reduced by all treatments, but no interaction effect could be detected. Potassium (K) was stronger reduced by nitrogen fertilisation in ambient air, as shown by the significant interaction term at 20 and 40 kg N ha^{-1} yr^{-1}. At the highest fertilisation level, the K concentration was reduced in all plants independent of the fumigation. Magnesium (Mg) concentrations showed a different reaction towards the treatments. With no interactive effects detectable, ozone fumigation significantly enhanced the Mg concentrations, which was also the case for the lowest nitrogen fertilisation. Forty kg N ha^{-1} yr^{-1} resulted in a slight increase of the Mg concentrations, whereas 80 kg N ha^{-1} yr^{-1} had no effect.

Table 5: Statistical analysis of the treatment effects of ozone fumigation (O_3) and nitrogen fertilisation (Nx) on nutrient concentrations and nutrient ratios according to the mixed effects linear model. The interaction term was only considered, where it was significant. Increasing or decreasing effects are indicated as „+“ or „-“ with indication of the significance level as (+), (-): $p < 0.1$; +, -: $p < 0.05$; + +, - -: $p < 0.01$; + + +, - - - : $p < 0.001$; n.s.: not significant. Data were corrected for soil pH. Observations in 4 groups, n = 75.

		O_3	**N20**	**N40**	**N80**
Nutrient concentrations					
N	main effects	n.s.	n.s.	n.s.	n.s.
P	main effects	- -	- -	- - -	- - -
K	main effects	n.s.	n.s.	n.s.	- -
	O_3 × N		(-)	- -	n.s.
Ca	main effects	n.s.	n.s.	n.s.	n.s.
Mg	main effects	+ +	+	(+)	n.s.
Nutrient ratios					
N : P	main effects	+	(+)	+ + +	+ + +
N : K	main effects	+ + +	n.s.	+	+ + +
N : Ca	main effects	n.s.	n.s.	n.s.	n.s.
N : Mg	main effects	-	-	(-)	n.s.

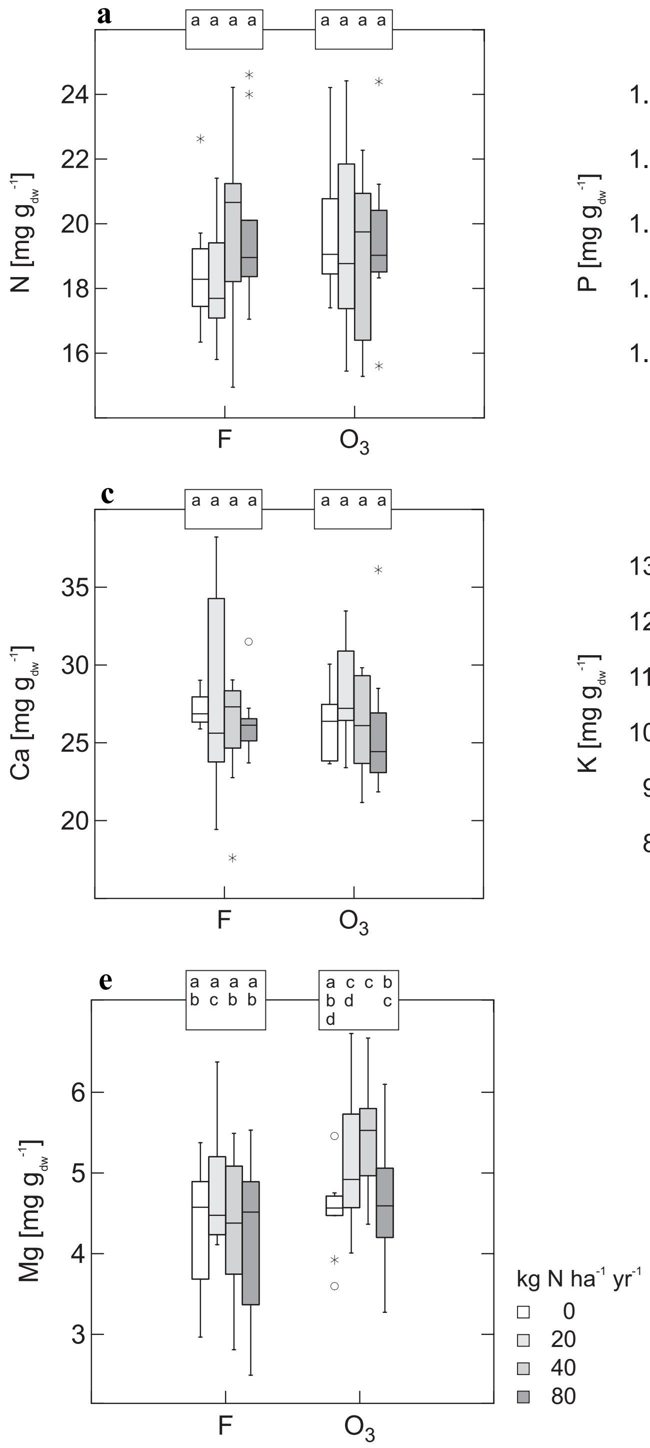

Figure 7: Effects of ozone fumigation and different nitrogen fertilisation treatments on nutrient concentrations in beech leaves. Given are a): nitrogen (N), b): phosphorus (P), c): calcium (Ca), d): potassium (K), and e): magnesium (Mg) concentrations. F: filtered air, O_3: ambient air. Differences ($p < 0.05$) are indicated by different letters. Statistics are given in Table 5.

4.4.6.2 Nutrient ratios

The treatments changed the nutrient ratios in similar ways, even though no interaction effects could be detected here (Table 5 and Figure 8). The N : P ratio was enhanced by ozone fumigation and by all nitrogen fertilisation levels, with only a slight increase at 20 kg N ha^{-1} yr^{-1}. For the N : K ratio the same effects were found, except that 20 kg N ha^{-1} yr^{-1} had no impact. A negative influence of ozone on the N : Mg ratio was found, which was also observed for 20 and 40, but not for 80 kg N ha^{-1} yr^{-1}.

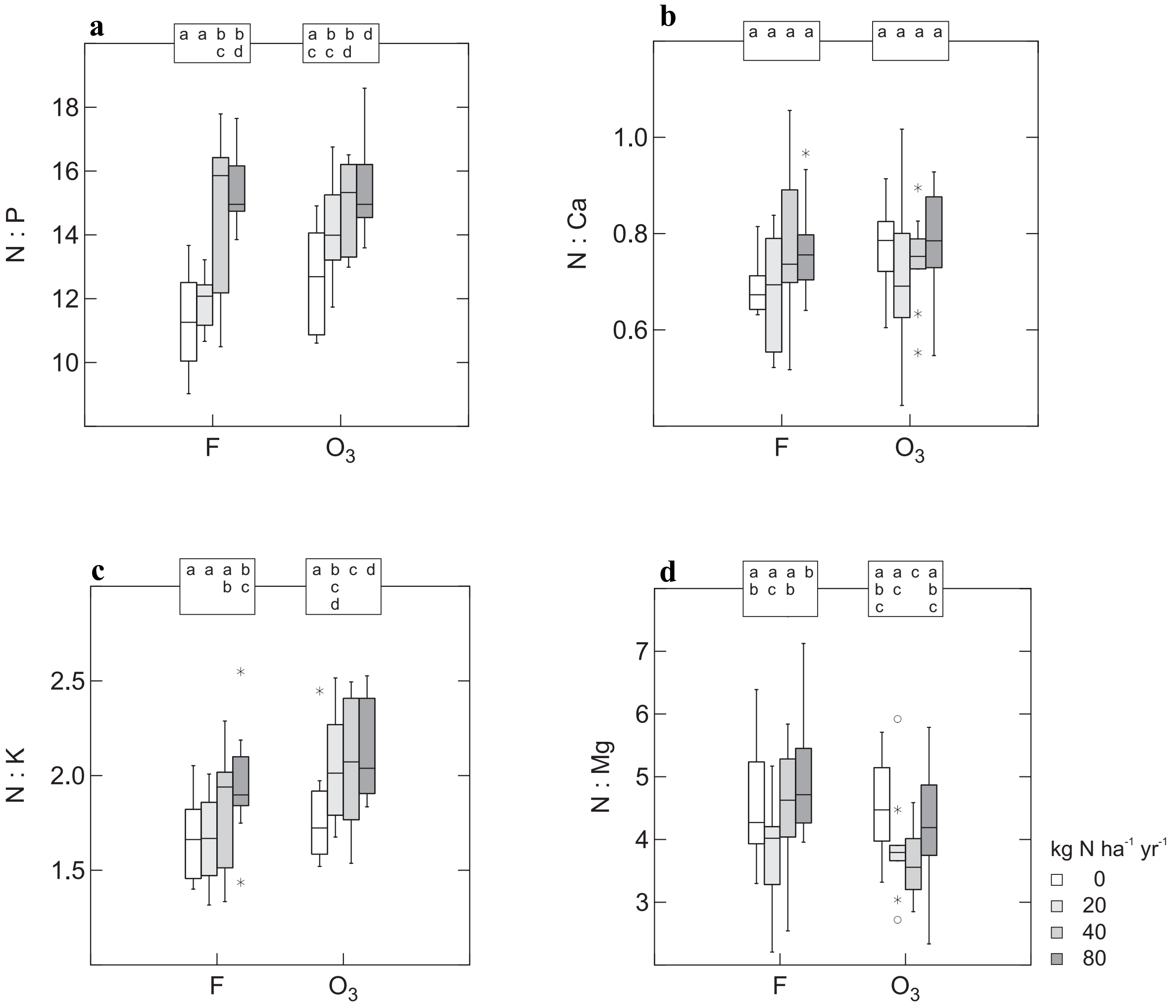

Figure 8: Effects of ozone fumigation and different nitrogen fertilisation treatments on nutrient ratios in beech leaves. Given are a): nitrogen : phosphorus (N : P), b): nitrogen : calcium (N : Ca), c): nitrogen : potassium (N : K), and d): nitrogen : magnesium (N : Mg) ratios. F: filtered air, O_3: ambient air. Differences ($p < 0.05$) are indicated by different letters, y-axes are square-root-transformed. Statistics are given in Table 4.

4.5 Discussion

A positive influence of nitrogen on plant growth for different species is well documented in the literature (Flückiger and Braun, 1999; Pääkkönen and Holopainen, 1995; Utriainen and Holopainen, 2001b). This was also observed in our experiment at nitrogen levels of 20 and 40 kg N ha^{-1} yr^{-1} for shoot elongation and aboveground biomass accumulation. The highest nitrogen load however, suppressed the mentioned parameters significantly. Also the leaf area of the beech seedlings was reduced at 80, but also at 20 kg N ha^{-1} yr^{-1}. These findings are different from the parallel study with spruce trees, where all nitrogen fertilisation levels enhanced elongation and biomass accumulation (Thomas et al., 2005). So far few studies described this negative effect of nitrogen fertilisation. Nellemann and Thomsen (2001) reported an initial growth stimulation followed by a subsequent decline for spruce trees in a large gradient study in Norway. In this case however, the decline might have been caused by an additional acidification effect, which could not be detected in our experiment, even though the soil pH started to decrease in the quarters receiving 80 kg N ha^{-1} yr^{-1} (Thomas et al., 2005). A growth reduction by nitrogen was also shown indirectly in the NITREX experimental plot in the Netherlands, where a reduction of the nitrogen deposition led to an increase in growth (Boxman et al., 1998). In accordance with our experiment, evidence has been provided that excessive nitrogen deposition can also inhibit root growth (Matzner and Murach, 1995). The observed changes in above- and belowground growth led to an increase in the shoot : root ratio at 20 and 80 kg N ha^{-1} yr^{-1}, which was also detected in other nitrogen fertilisation experiments by Flückiger and Braun (1998) or Pääkkönen and Holopainen (1995). An enhanced shoot : root ratio might leave plants more susceptible to drought. During our experiment, only short drought periods occurred in the second and third growth period. After these, leaf edge necroses developed in the beech saplings. The higher the nitrogen fertilisation, the more damages could be detected. The necroses could be due to the changes in the shoot : root ratios, but might also imply a lower water use efficiency for plants fertilised with high N loads. At least at 80 kg N ha^{-1} yr^{-1} and also in plants fumigated with ozone, lower leaf water contents were found, indicating a reduction in the water use efficiency of the beech seedlings. On the other hand, for 40 kg N ha^{-1} yr^{-1} the leaf water contents were increased, which is in contrast to the observed leaf necroses at this fertilisation level. Also leaf water potential measurements with a Scholander pressure chamber did not reveal any treatment differences (data not shown).

The effect of ozone on tree growth is ambiguous. Most studies describe decreases for shoot elongation and above- and belowground biomass accumulation (Momen et al., 2002; Reich and Lassoie, 1985; Stribley and Ashmore, 2002), but also increases (Spence et al., 1990; Yamaji et al., 2003) and no alterations (Alscher et al., 1989; Bortier et al., 2000a) have been reported. In our study a significant interaction of the nitrogen and ozone treatment on shoot elongation was found, which showed, that the ozone fumigation amplified the effect of the nitrogen fertilisation at 20 and 80 kg N ha^{-1} yr^{-1}. So far, few studies examined the combinatory effect of ozone fumigation and nitrogen fertilisation on growth, but in accordance with our results, the responses of *Pinus sylvestris* to elevated ozone were significantly modified by high nitrogen loads, leading to stronger growth inhibitions (Utriainen and Holopainen, 2001b). In our parallel experiment with *Picea abies* seedlings, no interactions on shoot elongation were found (Thomas et al., 2005), but a decrease in growth caused by ozone fumigation was also stated. In terms of biomass accumulation, *Populus tremuloides* plants proofed to be less responsive to ozone, when supplied with an excessive or even toxic supply of nitrogen (Pell et al., 1995). In our experiment, no combinatory effect was found for biomass accumulation, but a negative impact of the ozone fumigation on belowground biomass was revealed. Despite these findings, no changes of the shoot : root ratio occurred in plants fumigated with ozone. This is again different from the data obtained in the spruce experiment, where ozone fumigation did not change biomass accumulation above- or belowground, but decreased the shoot : root ratio (Thomas et al., 2005).

An interaction of ozone fumigation and nitrogen fertilisation was also detected for the LA, where the negative ozone and nitrogen impacts alleviated each other. Pell et al. (1995) found similar results for *Populus tremuloides* plants, where nitrogen could also attenuate the negative effects of ozone fumigation on LA. Even though no other interaction effects could be stated, ozone fumigation always had a negative influence on all leaf parameters investigated. Reports on the impact of ozone fumigation on different leaf parameters are not consistent. No effect as well as decreases in the LA or the water content have been stated for different tree species (Amundson et al., 1995; Pääkkönen and Holopainen, 1995; Shelburne et al., 1993).

Nitrogen fertilisation of 40 kg N ha^{-1} yr^{-1} and more enhanced the infestation of beech saplings with *Phyllaphis fagi* significantly. Previous findings have shown that nitrogen addition can lead to increased free amino acid concentrations in the leaves combined with decreases in phenolic compounds (Balsberg Påhlsson, 1992). This might be the reason for the observed susceptibility of nitrogen fertilised beech saplings towards aphid infestation, which was also stated in other experiments (Flückiger and Braun, 1999). Ozone fumigation did not affect the aphid infestation, which is in contrast to other observations (Braun and Flückiger, 1989; Holopainen et al., 1994). It should be stated however, that the nutrient concentrations and nutrient ratios were still within a normal range, which probably renders the plants more resistant towards aphid infestations. In addition, plants were not inoculated with aphids in a controlled way, but the infestation occurred "naturally" (Braun and Flückiger, 1989).

Nitrogen fertilisation at 80 kg N ha^{-1} yr^{-1} lead to an increase in monosaccharide, di- and trisaccharide, and total soluble carbohydrate concentrations in the finest roots, whereas the other nitrogen treatments did not cause any changes. The sugar alcohol concentrations were also enhanced at 80 kg N ha^{-1} yr^{-1}, but only in the plants fumigated with filtered air. In the parallel spruce study, nitrogen fertilisation also enhanced monosaccharide, di- and trisaccharide, as well as total carbohydrate concentrations, but the sugar alcohol concentrations were reduced. Few publications address the impact of nitrogen fertilisation on carbon allocation to roots. Grulke et al. (2001) found a significant decrease in the root monosaccharide concentrations in *Pinus ponderosa* with increasing nitrogen deposition. Wallenda et al. (1996) detected a decrease in the fungus specific compounds ergosterol, mannitol, and trehalose in *Picea abies* under an enhanced nitrogen supply, which was interpreted as a possible decrease in mycorrhization. Concentrations of trehalose and mannitol were also investigated in our experiment. For mannitol a significant decrease was found at 40 and 80 kg N ha^{-1} yr^{-1} ($p < 0.001$ each), but only for the plants fumigated with ozone (Figure 9), even though no combinatory effect was evident. The trehalose concentrations in the fine roots showed no clear response to the ozone or nitrogen treatment (data not shown). These results imply that the fertilisation did not simply increase or decrease the mycorrhization, but might have caused a change in the composition of the fungal symbionts. In contradiction to the soluble carbohydrate concentrations, the starch concentrations in the roots declined significantly at 80 kg N ha^{-1} yr^{-1}, whereas no other nitrogen treatments caused a change. Although starch and soluble carbohydrates were measured in different root fractions, the changes indicated a shift away from intermediate storage compounds. Especially, as the starch concentrations in stems were also significantly reduced at 80 kg N ha^{-1} yr^{-1}.

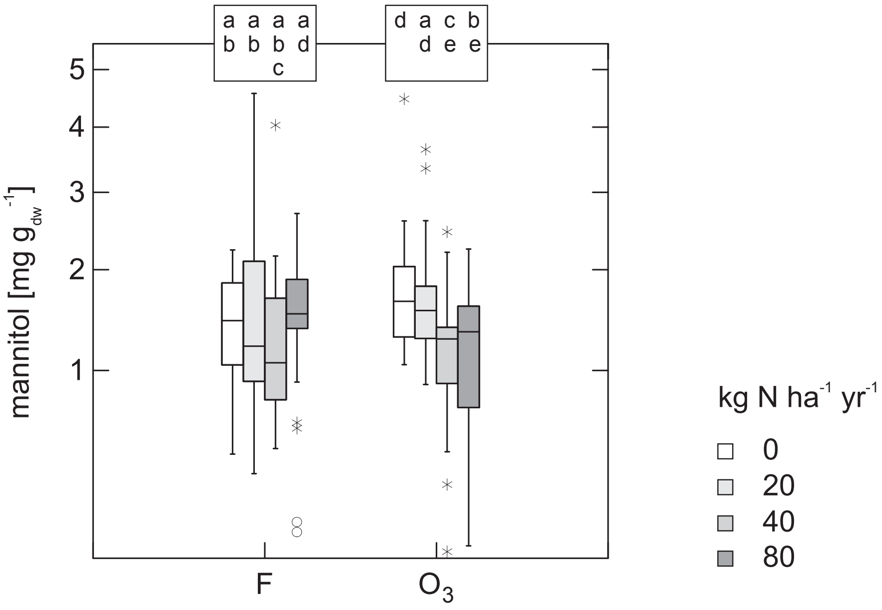

Figure 9: Effects of ozone fumigation and different nitrogen fertilisation treatments on mannitol concentrations in fine roots of beech seedlings. F: filtered air, O_3: ambient air. Differences ($p < 0.05$) are indicated by different letters, y-axes are root-transformed.

The ozone fumigation had similar effects, reducing the starch concentrations in the stems and the total amounts in roots. It has been suggested, that ozone impacts phloem loading and thus assimilate translocation from shoot to roots (Skärby et al., 1998), which can result in carbon accumulation in aboveground plant parts combined with a reduction in the roots. A significant reduction in root starch concentrations with increasing ozone concentrations was shown in a gradient study with mature *Fagus sylvatica,* but no aboveground plant parts were investigated (Thomas et al., 2002). Grulke et al. (2001) found decreased root and needle starch concentrations with simultaneous increases in bole starch concentrations in mature Ponderosa pine along an increasing ozone concentration and nitrogen deposition gradient. In our study however, starch concentrations decreased in roots and shoots. This was also shown in our parallel experiment for spruce trees, where starch concentrations declined in roots, stems, and needles under ozone fumigation (Thomas et al., 2005). Similar results were shown by Braun et al. (2004), where starch concentration in roots and stems of spruce seedlings declined along an ozone gradient, but needle starch concentrations increased only slightly. An overall decline in the starch concentrations could be explained, as photosynthesis is often impaired by ozone whereas respiration increases (Dizengremel, 2001). This may lead to an overall decrease in carbohydrate production. In addition, carbon metabolism is modified under ozone exposure for detoxification processes, protection and repair of the cell structure (Dizengremel, 2001).

Similar to the nitrogen effects on carbohydrates, the decreases in starch concentrations were accompanied by an increase in monosaccharide concentrations under ozone fumigation. The sugar alcohol and the di- and trisaccharide concentrations did not change. This is also similar to the spruce saplings, where only monosaccharide concentrations were enhanced, but not sugar alcohol or di- and trisaccharide concentrations (Thomas et al., 2005).

Although the nitrogen (N) concentrations in the beech leaves were not changed, the phosphorus (P) and potassium (K) concentrations declined significantly with increasing N fertilisation and thus also enhanced the N : P and N : K ratios. This is consistent with many other studies, including our parallel spruce experiment (Balsberg Påhlsson, 1992; Flückiger and Braun, 1998; Thomas et al., 2005), although for most tree species a significant increase in foliar N was also detected. Even though the observed nutrient ratios were still within the normal range (Flückiger and Braun, 2003), it is noteworthy, that within only three years of fertilisation the N : P ratio was shifted towards the upper limit of the recommended ratios. It has been suggested that a decrease of P might be caused by a reduced uptake as a result of soil acidification and hence aluminium (Al) toxicity (Haynes, 1982), or by a decreased supply rate of nutrients other than N due to leaching and N induced high growth rates, depleting the resource (Thelin et al., 1998). Both explanations are not likely for our experiment. The soil pH started to decline in the 80 kg N ha^{-1} yr^{-1} treatment, but the values were still high enough to exclude Al toxicity. At 80 kg N ha^{-1} yr^{-1} growth of the beech saplings was significantly reduced, so depletion is not possible. A negative impact of nitrogen deposition on mycorrhiza has also been reported (Arnolds, 1991; Wallenda and Kottke, 1998) and could be the cause for the observed changes in the nutrient concentrations. A change in the mycorrhization was already implied by changes in the mannitol concentrations but only for the ozone fumigated plants. The observed increase in the magnesium (Mg) concentrations was also reported for Scots pine by Utriainen and Holopainen (2001b). An addition of ammonia to the soil causes a release of cations to the soil solution, resulting in temporarily increased cation concentrations. The speed of the release is different for the individual cations. This might explain the enhanced concentrations of Mg in the leaves, whereas the K concentrations showed a decrease at the same time.

In our experiment ozone fumigation also had a significant impact on the P, K, and Mg concentrations and thus also affected the nutrient ratios. Changes in the nutrient concentrations caused by ozone fumigation have often been observed in different experiments, even though increases as well as decreases were stated for the individual nutrient concentrations (Utriainen et al., 2001; Utriainen and Holopainen, 2001a). These changes seem to depend on the soil properties and on the degree of the impact on plant metabolism of ozone fumigation (Reich and Schoettle, 1988). An impact of ozone fumigation on mycorrhization has been reported before (Edwards and Kelly, 1992; Gorissen et al., 1991), but the concentrations of the fungus-specific carbohydrates trehalose and mannitol concentrations were not significantly changed by the ozone fumigation in our experiment (mannitol: Figure 9, trehalose: data not shown).

4.6 Conclusions

Nitrogen fertilisation increased the susceptibility of the beech seedlings towards aphid infestation and drought, whereas the concentrations of phosphorus and potassium declined in the beech leaves, leading to higher nutrient ratios. Other parameters like shoot elongation, biomass accumulation, or starch concentrations were enhanced, but only for nitrogen loads of up to 40 kg N ha^{-1} yr^{-1}. In beech seedlings receiving the highest nitrogen fertilisation of 80 kg N ha^{-1} yr^{-1} a clear reversion of the positive effects could be detected, including shoot elongation, biomass accumulation, leaf water content, or starch concentrations. When these data were compared to the spruce data obtained in our parallel experiment, clear differences in the growth reactions of the two tree species could be revealed. In the spruce trees, all nitrogen fertilisation levels increased growth and biomass accumulation, without a reversion at 80 kg N ha^{-1} yr^{-1}. For the nutrient concentrations and nutrient ratios, similar patterns for both tree species could be observed, except that in spruce nitrogen concentrations in the needles were enhanced with nitrogen fertilisation.

Soluble carbohydrate concentrations in fine roots of beech increased significantly only at the highest nitrogen fertilisation level. A possible change in the mycorrhization of the beech seedlings with increasing nitrogen fertilisation is suggested, which is supported by changes in the fungus-specific sugar alcohol mannitol, but only for seedlings under ozone fumigation. These data are similar to the spruce data, where nitrogen fertilisation also enhanced soluble carbohydrate concentrations, except for the sugar alcohol concentrations, which were decreased in the fine roots.

The ozone fumigation resulted in reduced leaf area, leaf water content, shoot growth, root biomass accumulation, and decreased starch concentrations in beech. For spruce not all of these parameters were measured, but also shoot growth and starch concentrations in different plant parts were reduced under ozone fumigation.

Phosphorus and potassium concentrations declined in the beech leaves, increasing the N : P and N : K ratios, whereas in spruce trees ozone fumigation only affected the potassium concentrations and the N : K as well as the N : Ca ratios.

In beech, a combinatory effect of the two pollutants was found for leaf area and the shoot elongation, where ozone fumigation amplified the nitrogen effects. For spruce trees an antagonistic effect on the root starch concentrations was found, where higher nitrogen levels alleviated the negative impact of ozone.

We conclude that increasing nitrogen loads and ozone levels are likely to decrease tree health in forests via various impacts, including growth, biomass accumulation, changes in carbohydrate concentrations and nutrient ratios, and increased susceptibility towards drought and insect infestations. As a result of the two parallel experiments we also conclude that beech trees are more susceptible to enhanced nitrogen deposition compared to spruce.

4.7 Acknowledgements

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5. CHANGES IN ROOT STARCH CONCENTRATIONS OF MATURE BEECH (*FAGUS SYLVATICA* L.) ALONG AN OZONE AND NITROGEN GRADIENT IN SWITZERLAND

5.1 Abstract

Ozone and nitrogen deposition as anthropogenic pollution factors have been shown to affect carbon partitioning in plants. Both pollution factors have increased in recent decades exceeding the critical loads for forests in many places by now. Therefore, root starch concentrations of *Fagus sylvatica* L. were measured along an ozone and nitrogen gradient in Switzerland at 20 forest sites of mature trees. The starch concentrations were analysed enzymatically. We found a negative correlation for the root starch concentration with ozone and nitrogen, with an antagonistic interaction term, indicating that one pollution factor might alleviate the effect of the other.

5.2 Introduction

Ozone and nitrogen are two of the most important anthropogenic pollution factors for natural ecosystems such as forests. In recent years, ambient ozone doses often exceeded the critical level of 10 ppm h above AOT40 daylight (accumulated exposure over the threshold of 40 ppb) (Fuhrer et al., 1997). Ozone doses at these levels can impair carbon partitioning and thus, lead to a decrease of root carbohydrate concentrations (Cooley and Manning, 1987). Similarly, enhanced nitrogen loads are known to affect C-allocation as well (Wallenda et al., 1996). During the last decades, nitrogen deposition has increased exceeding the critical N load for forests of 10 - 20 kg N $ha^{-1}yr^{-1}$ in about 90 % of Swiss forests by now (Rihm, 1996).

Several belowground parameters, like carbohydrate reserves in roots, seem to be appropriate indicators for environmental changes. They are sensitive to stress and should be influenced prior to the occurrence of visible aboveground symptoms (Vogt et al., 1993). Particularly, Lux et al. (1997) have shown that ambient ozone doses (AOT40 daylight 12.3 and 19.6 ppm h) lead to a reduction in the root starch concentration of beech and spruce trees compared to filtered air. Similar effects were found by Wallenda et al. (1996) for spruce trees grown under enhanced N supply.

Both experiments were conducted on young trees being exposed to either enhanced ozone doses or nitrogen loads, but not a combination thereof. Since each factor plays an important role in the C-allocation of trees, it seems essential to explore carbohydrate fluxes and carbohydrate storage as a function of both atmospheric nitrogen input and ambient ozone levels. So far, only very few studies have taken this into account. Grulke et al. (2001)

examined the impact of both pollution factors on mature *Pinus ponderosa* trees in California, showing that root biomass and root starch concentrations were reduced at more polluted sites in comparison to cleaner ones. To our knowledge, no such studies have been conducted on mature deciduous trees in temperate regions so far. *Fagus sylvatica* L. is one of the main tree species in Switzerland and young trees are known to be sensitive to ozone (Braun and Flückiger, 1995) and nitrogen (Flückiger and Braun, 1999). Recent works also suggest an impact of nitrogen and ozone on the growth of mature beech (Braun et al., 1999). Therefore, we studied the effect of both pollution factors on the root starch concentration of mature beech trees in Switzerland.

5.3 Materials and Methods

We selected 20 forest sites of mature beech with presumably acidic soil conditions below 1100 m asl along an ozone and nitrogen gradient in Switzerland.

Using the nitrogen deposition model of Rihm and Kurz (2001), calculated N-loads varied between 29 and 71 kg N ha^{-1} yr^{-1} for the respective site (Figure 1). In 1999, modelled ozone doses (Achermann and Rihm, 1997) ranged from 7 to 36 ppm h AOT40 daylight (accumulated ozone dose over the threshold of 40 ppb) (Figure 1). In January and February 2000 we sampled roots and adhering rhizospheric soil in two different soil depths (upper soil layer: 0 - 15 cm and lower soil layer: 35 - 60 cm) of six to seven trees per site.

The adhering soil was carefully removed from the roots and dried at 40 °C. For the pH-analysis 15 ml of a $CaCl_2$-solution (0.01 M) were added to 6.0 g of the dried soil. Samples were shaken at regular intervals and the pH of the solution was measured after at least one hour, using a double junction glass electrode (713 pH Meter, Metrohm, Herisau, Switzerland).

Roots were washed with cold water and treated with ultrasonic waves to clean them carefully. Medium-sized root parts (diameter: 1.0 - 2.5 mm) were selected for starch analysis. After rinsing them twice with demineralised water they were padded dry with tissue-paper, shock-frozen in liquid nitrogen and finally freeze-dried (Pirani, Edwards High Vacuum, Crawley, England). Dried samples were ball-milled (Retsch MM2, Retsch GmbH & Co KG, Haan, Germany) and stored at – 20°C in plastic bags over silica gel until further analysis.

The starch concentration of the freeze-dried roots was analysed enzymatically as described by Boehringer-Mannheim (1989). Forty mg of the powdered plant material were washed three times with 1 ml ethanol (80 % v/v) to remove mono- and oligosaccharides. The remaining pellet was dried again (Speed Vac Plus SC 110 A, Savant Instruments Inc., Holbrook, NY) and digested for 30 min at 60 °C using 1 ml of dimethylsulfoxide/HCl (8 M) in a 4:1 (v/v) ratio. After neutralization of the digest, starch was hydrolysed with amyloglucosidase from *Aspergillus niger* (Boehringer, Mannheim, Germany). Free glucose was assayed using hexokinase/glucose-6-phosphate-dehydrogenase as described by Boehringer-Mannheim (1989).

For statistical analyses SYSTAT 9 (SYSTAT Inc., Evanston, IL, USA) and S-PLUS 2000 (MathSoft Inc., Cambridge, MA, USA) were used. The effect of N-deposition and ozone-dose on the root starch concentration was quantified using a linear mixed effects model with the site as a grouping variable. Differences in soil pH and starch concentration of the roots between soil layers were determined by a t-test for dependent samples. The residuals of the tests were checked for normal distribution.

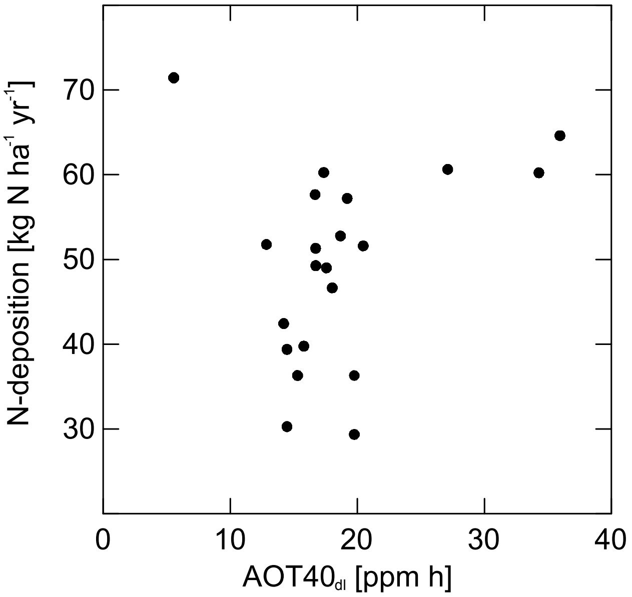

Figure 1. Ozone concentration and nitrogen deposition for the study sites. Each dot represents one study site (n = 20).

5.4 Results

The rhizospheric soil was acidic for the selected sites with even more acidic pH values in the lower: 3.9 ± 0.1 (mean ± SE) than in the upper soil layer: 4.1 ± 0.1 (mean ± SE). Differences are significant at $p < 0.001$, df = 119 (t-test for dependent samples). Starch values of individual root samples range from 3 to 68 mg g_{dw}^{-1} irrespective of the soil layer (Figure 2).

Ozone and nitrogen loads correlated negatively with the root starch concentrations of both soil layers (Table 1). Both pollution variables lead to a significant reduction of the root starch concentration showing a much stronger correlation with the ozone dose in comparison to the nitrogen load. The interaction term indicated that the effect was strongest for low and medium pollution situations (defined as: below 25 ppm h > AOT40 daylight and below 60 kg N ha^{-1}

yr^{-1}), whereas for sites with high ozone and nitrogen loads the effect got weaker. Root starch concentration was not affected by soil pH in either soil layer.

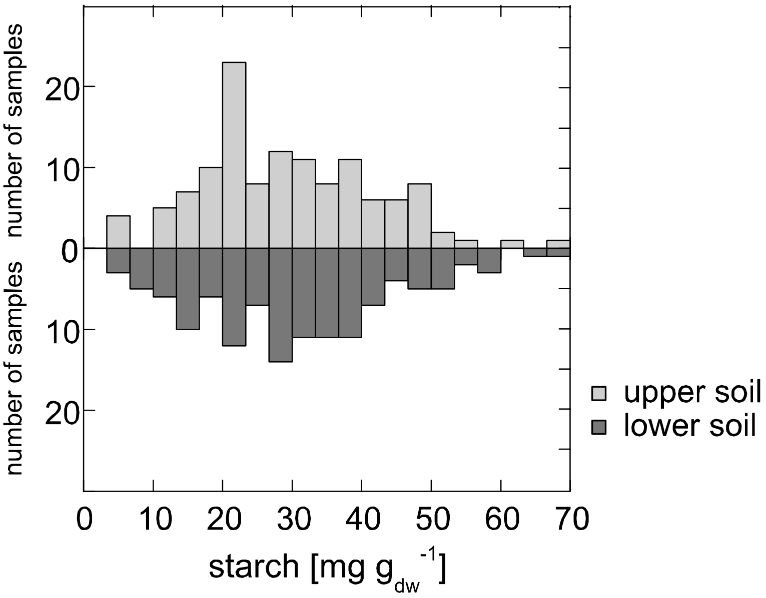

Figure 2: Root starch concentrations of the two soil layers (upper soil layer: 0 – 15 cm, lower soil layer: 35 – 60 cm).

Table 1: Regression parameters for root starch concentrations of the upper and lower soil layer. Modelled ozone data for the year 1999 and modelled nitrogen deposition (N-depo) were used. For each soil layer: number of observations: 124; number of groups: 20 sites. SE = standard error.

	Coefficient	SE	p-value
Upper soil layer			
(Intercept)	124.154	31.487	< 0.001
ozone 99	-6.044	1.987	0.0078
N-depo	-1.411	0.494	0.0115
N-depo × ozone 99	0.093	0.031	0.0094
Lower soil layer			
(Intercept)	155.701	34.304	< 0.001
ozone 99	-8.076	2.165	0.0018
N-depo	-1.791	0.539	0.0043
N-depo × ozone 99	0.120	0.034	0.0029

5.5 Discussion

Root starch concentration of the investigated mature beech trees was a negative function of both ozone dose and nitrogen load. However, ozone had a much stronger effect than nitrogen. Also the combination of the two pollution factors was significantly correlated with root starch concentration. The positive value of the interaction term indicated that the combined effect of ozone and nitrogen was strongest for sites with low to medium pollution values. For the combination of high nitrogen loads and high ozone doses the impact was much weaker, indicating that one pollution factor might alleviate the effect of the other. These findings are consistent with the results of some studies with deciduous tree seedlings. For example Pääkkönen and Holopainen (1995) found that sufficient nitrogen supply can confer young *Betula pendula* with greater resistance to ozone. In another study, *Populus tremuloides* seedlings were grown with an excess or even a toxic supply of nitrogen. They also tended to be less responsive to ozone in terms of growth rates (Pell et al., 1995). Despite these findings and the notion of one factor alleviating the effect of the other, the root starch concentrations for the high pollution groups in our experiment were still below the levels of sites with low nitrogen deposition and low ozone concentrations. Similar to our results, Grulke et al. (2001) found reduced root starch concentrations in mature *Pinus ponderosa* trees at sites with high ozone and high N deposition compared to cleaner sites. In contrast, the bole starch concentration of *Pinus ponderosa* was significantly increased at the most polluted sites (Grulke et al., 2001).

However, the mechanism behind the observed carbon partitioning due to enhanced ozone doses or nitrogen loads is not yet fully understood. As an effect of ozone, Bortier et al. (2000b) suggested carbon retention in the leaves for repair of damaged foliage and a decreased phloem loading. For nitrogen, a decreased root starch concentration might be due to a reduced carbon translocation from the shoot together with a higher need for carbon skeletons for amino acid synthesis under increased inorganic nitrogen supply (Wallenda et al., 1996). Why one factor might alleviate the other under high pollution conditions remains open and needs further investigations.

A reduced root starch concentration might coincide with other effects for the trees. According to Ziegler (1991), starch storage in roots is an overall sensitive indicator for the status of carbohydrate reserves in a tree. Elevated ozone doses might result in a higher carbon retention in the shoot for ozone detoxification and lower carbon allocation to the roots leading to alterations in whole tree biomass after several years (Takemoto et al., 2001). As a consequence, trees might become more susceptible to stresses like drought, windthrow, and root diseases (Takemoto et al., 2001). Similar findings are stated for an enhanced nitrogen deposition. Besides a lowered carbon allocation to the roots (Wallenda et al., 1996) the fine root production is decreased (Nadelhoffer, 2000) and shoot : root ratio increased (Flückiger and Braun, 1998) with a higher nitrogen availability. Cooley and Manning (1987) provided evidence, that changes in carbohydrate partitioning may even affect the way plants interact with other organisms, particularly symbionts, but also pathogens. This has been shown for ozone (Andersen and Rygiewicz, 1995) and also for an enhanced nitrogen supply (Wallenda et al., 1996).

The change in carbon allocation resulting in a reduced root starch concentration in mature beech trees may lead to various additional effects. Enhanced ambient ozone concentrations and nitrogen supply as they can be observed in Switzerland might play an important role in tree health of *Fagus sylvatica* L.

5.6 Acknowledgements

We would like to thank B. Rihm for his support with the ozone and nitrogen modelling, as well as R. Haas and M. Tobler for helping with the root sampling. This work was funded by the Swiss Agency for the Environment, Forests and Landscape (SAEFL).

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6. ROOT STARCH CONCENTRATIONS IN MATURE SPRUCE TREES (*PICEA ABIES* [L.] KARST.) ALONG AN OZONE AND NITROGEN GRADIENT IN SWITZERLAND

6.1 Abstract

Twenty-one sites of mature spruce trees were chosen along a gradient of nitrogen depositions and ozone doses in Switzerland. Roots of six trees per site were excavated in winter 2001 and starch concentrations of medium-sized roots were analysed enzymatically. Both pollutants decreased root starch concentrations significantly. A positive interaction term indicated, however, that the two pollutants alleviated the effect of each other. This was shown for ozone doses above 18 ppm h > 40 ppb and nitrogen depositions > 35 kg N ha^{-1} yr^{-1}. The significant decreases in root starch concentrations indicate an important impact of both pollutants on forest tree health. Furthermore, the obtained results suggest that mature trees are more susceptible towards the two pollutants than seedlings.

6.2 Introduction

Roots are highly sensitive plant organs that often show reactions towards pollutants earlier than aboveground plant parts. Especially parameters like fine root biomass, carbohydrate reserves, secondary defensive chemical compounds, or nutrient concentrations have a high potential as indicators for anthropogenic stresses (Vogt et al., 1993). For example ozone exposure of plants results in reduced carbon assimilation and altered carbon partitioning, with roots being affected prior to aboveground plant parts (Andersen, 2003). Due to impaired phloem loading, carbohydrates remain in shoots or leaves, whereas root carbohydrate concentrations decline (Cooley and Manning, 1987).

Similar, nitrogen fertilisation of up to 80 kg N ha^{-1} yr^{-1} has been found to decrease concentrations of storage compounds like starch in beech seedlings (Thomas et al., 2005). Wallenda et al. (1996) revealed a decline in root carbohydrate concentrations under nitrogen fertilisation, suggesting a shift of carbon flow from starch formation towards an enhanced provision of carbon skeletons for N assimilation and shoot growth. As both pollutants impact carbon allocation and as a consequence reduce root starch concentrations, the examination of simultaneous ozone exposure and enhanced nitrogen deposition is highly necessary. Even a simple additive effect of the two pollutants could reduce root starch concentrations drastically. Alterations in whole-tree biomass are likely and as a consequence, susceptibility to drought, windthrow, and root diseases could be exacerbated for trees (Takemoto et al., 2001).

For mature beech, a significant reduction of root starch concentrations under enhanced nitrogen deposition and ozone exposure has already been stated in a similar gradient study (Thomas et al., 2002), although, the combination of the two pollutants resulted in an alleviation of the negative effects at high pollution levels. However, beech is the only tree species of temperate forests, where the combinatory effects have been studied on mature trees so far. To gain knowledge on possible impacts on forest health under increased pollution levels, more information on different tree species are of great importance. Therefore we chose mature *Picea abies* as study species, which is the major conifer tree species in Switzerland. It is particularly necessary to reveal combinatory effects of nitrogen deposition and ozone exposure on forest trees, because both pollutants frequently exceed the critical levels ($AOT40_{dl}$ 5 ppm h) and loads (10 – 20 kg N ha^{-1} yr^{-1}) for forests in Switzerland (Fuhrer et al., 1997; Rihm, 1996).

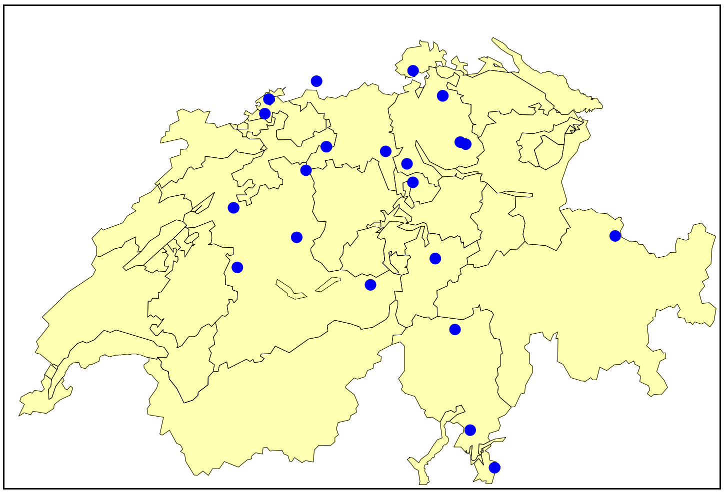

Figure 1: Distribution of selected study sites with mature spruce in Switzerland. All sites are located on acidic soil, below 1100 m asl.

6.3 Materials and Methods

We selected 21 forest sites of mature spruce with presumably acidic soil conditions below 1100 m asl along an ozone and nitrogen gradient in Switzerland (Figure 1). Modelled N-loads varied from 17.1 to 48.1 kg N ha^{-1} yr^{-1} and modelled ozone doses ($AOT40_{dl}$) ranged from 6.4 to 39.5 ppm h > 40 ppb in the year 2000 for the selected spruce sites. Nitrogen loads and ozone doses for the different sites are shown in Figure 2.

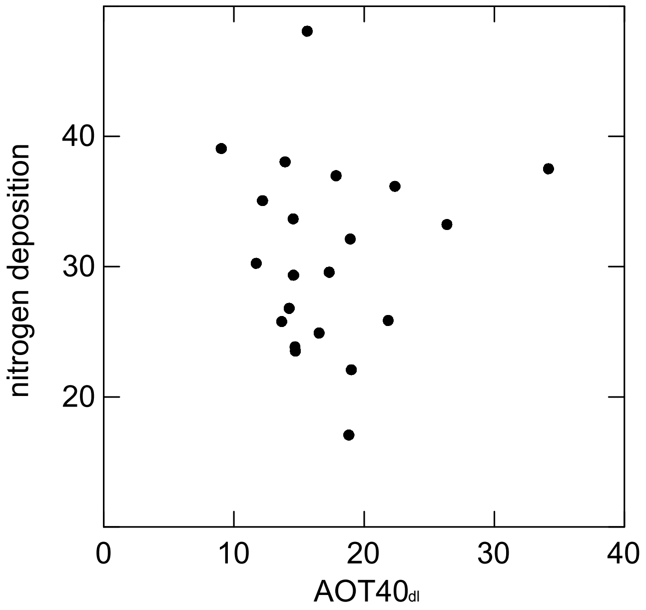

Figure 2: Modelled ozone doses for the year 2000 [ppm h > 40 ppb] and nitrogen depositions [kg N ha^{-1} yr^{-1}] for the selected spruce sites.

In January and February 2001 we sampled roots of six spruce trees per site from one soil layer (0 – 30 cm). The adhering rhizospheric soil was collected, dried at 40 °C for two days and pH was measured electrometrically in a 1 : 2.5 suspension with $CaCl_2$-solution (0.01 M) (713 pH Meter, Metrohm, Herisau, Switzerland). Roots were washed with cold water and medium-sized root parts (diameter: 1.0 - 2.5 mm) were selected for starch analysis. Thereafter, they were shock frozen in liquid nitrogen, lyophilised, and ball-milled, as described in detail by Thomas et al. (2002).

Forty mg of the ground plant material were washed with demineralised water to remove soluble carbohydrates. The starch concentrations were analysed enzymatically and measured photometrically, as described in detail by Thomas et al. (2002) and Boehringer (1989).

For statistical analyses the software packages SYSTAT 10 (Systat Inc., Evanston, IL, USA) and S-PLUS 2000 (MathSOFT INC., Cambridge, MA, USA) were used. Starch concentrations were root-transformed for analyses. Effects of nitrogen deposition and ozone dose as well as their possible interaction were quantified using a linear mixed effects model with sites as a grouping variable. The residuals of the tests were checked for normal distribution and the presence of outliers.

6.4 Results

The rhizospheric soil from the chosen sites showed pH-values between 2.9 ± 0.1 and 5.5 ± 0.3 (mean ± SE). Across the different sites, root starch concentrations ranged from 32.66 ± 4.98 mg g_{dw}^{-1} to 112.78 ± 7.24 mg g_{dw}^{-1} (mean ± SE). Increasing nitrogen deposition and ozone dose resulted in a decrease of the root starch concentrations. The combination of the two pollutants revealed an antagonistic effect (Table 1). After discriminance analysis, the pollution levels and loads were divided into groups for visualisation of the interaction effects. For pollution levels below an $AOT40_{dl}$ of 18 ppm h the decrease in root starch concentration with increasing nitrogen deposition was significant, whereas for sites with ozone levels above an $AOT40_{dl}$ of 18 ppm h no reductions could be stated (Figure 3). Similarly, for nitrogen deposition loads below 35 kg N ha^{-1} yr^{-1} (given in two groups: < 25 and ≥ 25 to < 35 kg N ha^{-1} yr^{-1}) decreases in root starch concentrations were found with increasing ozone doses. At high loads of more than 35 kg N ha^{-1} yr^{-1}, however, no decline in root starch concentrations could be detected (Figure 4).

Table 1: Regression parameters for the spruce root starch concentrations. Root starch concentrations were root-transformed for calculation. Modelled ozone data for the year 2000 were used. Number of observations: 124; number of groups: 21 sites.

	Coefficient	Std Error	p-value
(Intercept)	25.647	5.108	< 0.001
ozone	- 1.027	0.299	0.003
N-deposition	-0.506	0.145	0.003
N-deposition × ozone	0.028	0.008	0.004

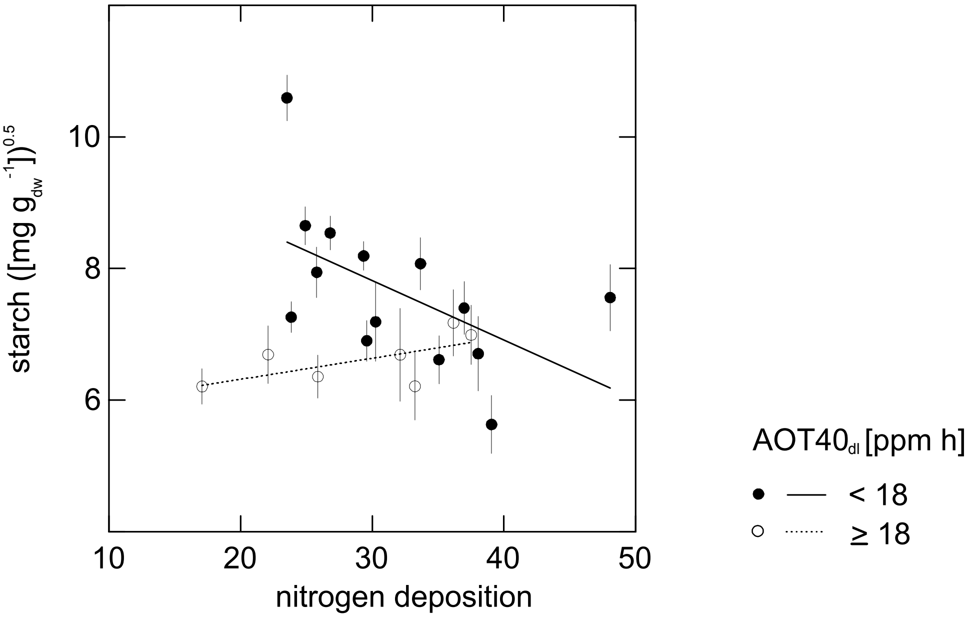

Figure 3: Root starch concentrations along the modelled nitrogen gradient. Nitrogen deposition is given in [kg N ha^{-1} yr^{-1}]. Ozone doses are shown in two groups: < 18 and ≥ 18 ppm h > 40 ppb. Root starch concentrations are root-transformed. Observations at 21 sites, n = 126.

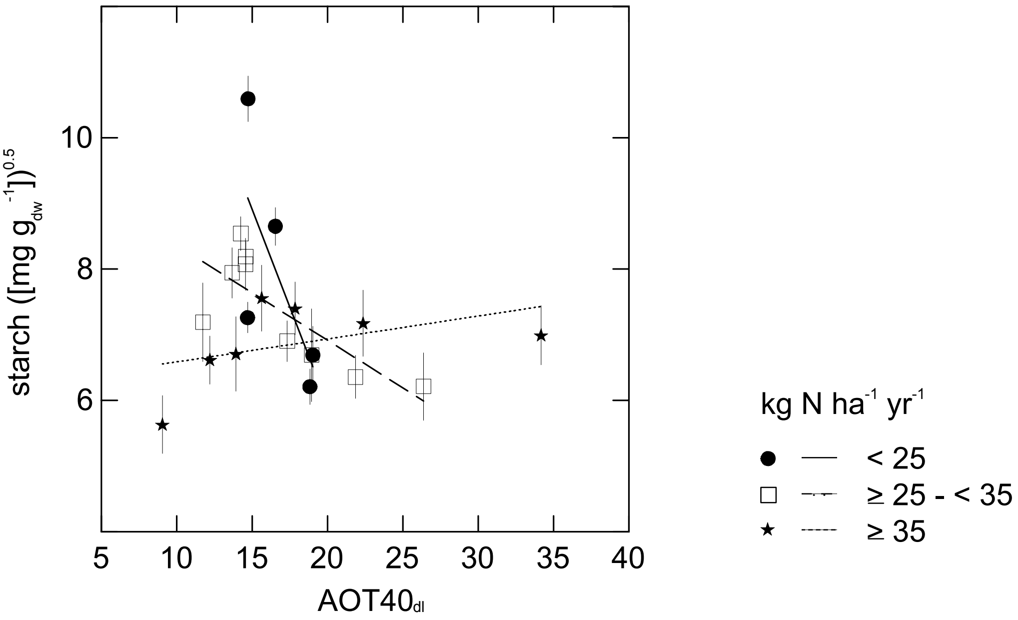

Figure 4: Root starch concentrations along the modelled ozone gradient. Ozone doses are given in ppm h > 40 ppb. Nitrogen deposition is shown in three groups: < 25, ≥ 25 to < 35, and ≥ 35 deposition rates in [kg N ha^{-1} yr^{-1}]. Root starch concentrations are root-transformed. Observations at 21 sites, n = 126.

6.5 Discussion

Ozone and nitrogen exposure reduced the root starch concentrations of mature spruce trees. These findings are in consistence with a study of Grulke et al. (2001) who found reduced carbohydrate concentrations in roots of mature *Pinus ponderosa* trees with increasing nitrogen and ozone pollution. Especially ozone is thought to reduce root carbohydrate concentrations caused by an impaired phloem loading (Skärby et al., 1998). As a consequence root starch concentrations decline, whereas carbohydrate concentrations in aboveground plant parts like shoots or needles may be enhanced. This was shown for mature Ponderosa pine, where bole starch concentrations increased with increasing nitrogen loads and ozone levels (Grulke et al., 2001). However, Braun et al. (2004) found decreases of root starch concentrations in roots as well as in shoots of spruce seedlings along an ozone gradient, with only slight increases in the needles. In a three-year fumigation and fertilisation experiment with young spruce, ozone led to decreases of starch concentrations in roots, shoots, and also needles (Thomas et al., 2005). This indicates that probably fewer carbohydrates are available for storage in trees fumigated with ozone. Also Dizengremel et al. (2001) suggested that decreases in photosynthesis together with an enhanced need of carbohydrates for repair mechanisms cause generally lower concentrations of storage carbohydrates in trees under ozone exposure. Similar reductions in root carbohydrate concentrations were shown for spruce seedlings under enhanced nitrogen fertilisation (Wallenda et al., 1996). A higher need of carbon for an enhanced shoot growth was suggested. These results are in consistence with our findings of decreased root starch concentrations with increasing nitrogen depositions. Spruce seedlings fertilised with nitrogen for three years, however, have shown slightly enhanced root starch concentrations (Thomas et al., 2005). In this experiment fertilisation levels of up to 80 kg N ha^{-1} yr^{-1}, with a modelled background deposition of 15 kg N ha^{-1} yr^{-1}, were used, whereas in the gradient study, nitrogen loads reached levels of up to 48 kg N ha^{-1} yr^{-1}. The mature spruce trees have received less nitrogen in terms of annually loads, but over a longer time period than the seedlings. This indicates that mature spruce trees might be more sensitive to the combination of the two pollutants compared with seedlings. For ozone exposure alone, this has been shown for seedlings and mature trees of *Quercus robur* (Samuelson and Kelly, 1996). In their experiment, starch concentrations decreased in leaves and increased in branches of mature individuals, whereas in seedlings the concentrations remained unaffected under ambient ozone exposure.

In our study, both pollutants reduced the root starch concentrations significantly, but the interaction term was positive. This indicated an alleviation of the effects at high pollution concentrations of more than 18 ppm h > AOT40$_{dl}$ and more than 35 kg N ha^{-1} yr^{-1}. In a similar gradient study with mature beech trees comparable results were obtained (Thomas et al., 2002), where increasing ozone levels and nitrogen loads also decreased root starch concentrations, with an alleviation of the effects for high pollution concentrations. In an experiment with *Betula pendula*, greater resistance towards ozone exposure was found, when the seedlings were supplied with 74 or 150 kg N ha^{-1} yr^{-1} (Pääkkönen and Holopainen, 1995). Also growth rates of *Populus tremuloides* seedling were less affected by ozone exposure when supplied with an excess or even a toxic supply of nitrogen (Pell et al., 1995).

Despite the observed alleviation of effects at high pollution levels, a general decline of root starch concentrations with increasing ozone levels and nitrogen loads has to be stated. This might further lead to reductions in root growth and biomass. As a consequence increased susceptibility to drought, windthrow, and root diseases are to be expected (Takemoto et al., 2001).

The currently observed ozone levels and nitrogen loads are therefore likely to play an important role in forest tree health in Switzerland.

6.6 Acknowledgements

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7. SUMMARY AND CONCLUSIONS

Tropospheric ozone (O_3) and nitrogen deposition (N) are considered as two of the most important anthropogenic pollution factors for temperate forests today. Even though many studies demonstrate the impact of either one of the two pollutants on forest trees, little is still known on combinatory effects. However, as both, ozone concentrations and nitrogen depositions exceed the critical levels and loads for forests in Switzerland it is essential to investigate the impacts of a simultaneous exposure of trees to these two pollutants. Both, the scarcity and the need for further information in that respect motivated this thesis.

To investigate the combinatory effects on forest trees, the two main tree species of Switzerland, *Fagus sylvatica* and *Picea abies*, were chosen as study species – seedlings as well as mature trees.

From literature we know that similar parameters are affected by either N or O_3 exposure, e.g. shoot elongation, biomass accumulation, or root carbohydrate concentrations. Regarding these parameters trees might react to concurrent exposure in a simple additive way, i.e. positive and/or negative trends sum up to a total effect. In this case individual effects do not interact. Adversely, O_3 and N effects interact, which may lead to either aggravation or alleviation of the sum of individual effects.

I chose two different approaches to reveal possible interactions but also to elucidate the individual effects of trees being exposed to the two pollutants N and O_3:
a) A three-year controlled fumigation (O_3) and fertilisation (N) experiment with tree seedlings was conducted to investigate allometrical, physiological, and phenological parameters.
b) Mature trees along an O_3 and N gradient were tested for changes in root carbohydrate concentrations.

For spruce seedlings a significant interaction of the two pollutants was found for the root starch concentrations where the negative effect of O_3 counteracted a positive effect of N in an antagonistic way. Other combinatory effects were mainly additive, without any interaction effect detectable: Monosaccharide concentrations of fine roots were raised by both N-fertilisation (+ 10 %) and O_3 fumigation (+ 10 %). Also the formation of needle discolorations (chlorotic mottling) was increased by both pollutants. Conversely, shoot elongation and starch concentrations of stems were enhanced by N but counteracted by O_3, without interaction of the two pollutants. Trehalose concentrations also showed an additive effect, but here, nitrogen fertilisation showed a decreasing and ozone fumigation an increasing trend. For the nutrient concentrations and nutrient ratios an additive effect was found for potassium (both negative) and the N : K ratio (both positive).

In other parameters measured, only one of the two treatments was responsible for the observed changes: Increased N levels resulted in higher above- and belowground biomass accumulation, without a shift of the shoot : root ratio. On the other hand, this ratio was influenced by O_3 (- 10 %) although neither above- nor belowground biomass was significantly affected by ozone fumigation. Also soluble carbohydrates in spruce fine roots (except monosaccharides; see above) were mainly influenced by N alone: Sugar alcohol concentrations, including mannitol, decreased (- 17 %) while di- and trisaccharides concentrations were elevated (+ 10 %) with increasing N. Furthermore, the nutrient concentrations and ratios in the needles showed mostly N effects: N concentration increased, whereas P, Mg, and Mn concentrations decreased, leading to enhanced N : P, N : K, and N : Mg ratios.

Beech seedlings showed similar patterns in their reaction to N and O_3 treatment compared with spruce seedlings, however, beech tended to be more sensitive to higher N loads. In general, I found supportive fertilisation effects with increasing N up to 40 kg N ha^{-1} yr^{-1} which were reversed for the highest level of 80 kg N ha^{-1} yr^{-1} compared to controls. This reaction of beech seedlings makes description of results somewhat more complicated.

Despite this complexity I found statistically comprehensible interaction effects of N and O_3, e.g. in shoot elongation, leaf area, or sugar alcohols. In most cases, these significant interactions were characterised by an amplification of the N effects by O_3 - in positive as well as in negative ways. To exemplary illustrate this statement I describe the results for the shoot elongation in some detail: at 20 kg N ha^{-1} yr^{-1} elongation was significantly increased (F: + 4 %). This nitrogen-induced change was amplified in plants fumigated with ozone (O_3: + 19%), as was shown by a positive interaction term. Nitrogen fertilisation of 40 kg N ha^{-1} yr^{-1} increased shoot growth in all plants (F: + 17 and O_3: + 13 %), independent of the fumigation. For the highest fertilisation level, however, nitrogen reduced shoot elongation below that of controls (F: -8 %), which was again amplified in plants fumigated with ozone (O_3: - 28 %).

In various parameters I detected simple additive effects of the two pollutants: e.g. biomass accumulation belowground, leaf water content, specific leaf area, starch amounts in shoots and roots, starch concentrations in stems, monosaccharide concentrations in fine roots, nutrient concentrations (P, K, Mg), and nutrient ratios N : P, N : K, N : Mg.

Finally, also in beech seedlings I found parameters that were only influenced by one of the two pollutants, namely nitrogen: e.g. leaf necroses, aphid infestation, biomass accumulation aboveground, shoot : root ratio, and di- and trisaccharide concentrations.

When comparing the results of the two tree species, beech seedlings seemed to be more susceptible towards N than spruce trees. This was mainly indicated by the reversion of the "positive" N effects at the highest fertilisation level of 80 kg N ha^{-1} yr^{-1} for beech. Although the sensitivity of beech and spruce seedlings towards O_3 did not seem to differ considerably the interaction effects did. In spruce, the only interaction effect detected, was an antagonistic one for starch concentrations in coarse roots. In beech however, ozone amplified the nitrogen effects in positive, as well as in negative ways (e.g. shoot elongation).

In the gradient study on mature trees, similar effects of O_3 and N exposure on root starch concentrations of beech and spruce were detected. In both tree species root starch concentrations declined with increasing nitrogen and ozone concentrations. An antagonistic interaction term for the two pollutants was found, which indicated that the two pollutants alleviated the effect of each other at high concentrations, in beech as well as in spruce trees.

Comparing seedlings and mature trees in their reaction towards the combination of the two pollutants the studies indicate a higher sensitivity of mature trees. Significant decreases in the root starch concentrations under elevated O_3 and N, as well as a significant interaction were found. In the beech seedlings root starch concentrations did not decline significantly under ozone fumigation. However, ozone concentrations in the fumigation experiment were relatively low compared to the sites used in the gradient study (ozone fumigation: $AOT40_{dl}$ 7.2 – 22.1 ppm h; gradient study: $AOT40_{dl}$ 5.6 – 36.0 ppm h). Mature spruce showed the same reaction pattern like mature beech: root starch concentrations decreased with increasing pollution concentrations, showing a significant antagonistic interaction. In the spruce seedlings, however, root starch concentrations increased with nitrogen fertilisation, which contrasts the observations with mature trees. But here again, the ozone concentrations in the fumigation experiment were lower, compared to the sites in the gradient study (ozone fumigation: $AOT40_{dl}$ 7.2 – 22.1 ppm h; gradient study: 6.4 to 39.5 ppm h). Overall the findings suggest a higher sensitivity of mature trees of both species, compared with seedlings.

7.1 Main statements and conclusions

- The interaction effects of the two pollutants are far from being uniform. The combination of ozone and nitrogen results in amplification (e.g. shoot elongation in beech seedlings) or alleviation (e.g. root starch concentrations in mature spruce and beech) of the effects, whereas some parameters are subject to additive "interactions" (e.g. shoot elongation in spruce seedlings), or are only influenced by one of the pollutants (e.g. biomass in spruce seedlings (N)).
- As an overall effect of nitrogen fertilisation, shoot elongation, aboveground biomass accumulation (at least up to 40 kg N ha^{-1} yr^{-1}), and monosaccharide concentrations are enhanced, whereas phosphorus concentrations in needles and leaves decrease. Ozone fumigation has general decreasing effects on shoot elongation and also on starch concentrations.
- Beech and spruce seedlings react similar towards ozone and nitrogen exposure, but beech seems to be more sensitive, especially towards nitrogen fertilisation.
- Mature trees react stronger in their response towards ozone and nitrogen exposure, concerning root starch concentrations. This supports the notion that mature trees might be more sensitive towards the two pollutants than seedlings.
- Ozone exposure and nitrogen fertilisation induce changes in the fungus-specific sugar alcohols mannitol and trehalose, which suggest impacts on the mycorrhization of the seedlings.
- The observed changes in the nutrient concentrations and nutrient ratios are concerning, especially as they are likely to proceed further under ongoing and probably more severe exposure. Particularly phosphorus deficiencies might become crucial for tree health.
- Due to the detected changes in the investigated parameters and the observed needle discolorations, leaf necroses, and aphid infestations it is very likely that tree health in general will decrease under simultaneous ozone and nitrogen exposure.

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9. ABBREVIATIONS

c + unit	centi
k + unit	kilo
M + unit	mega
m + unit	milli
µ + unit	micro
n + unit	nano

AAS	atomic absorption spectrometer
Al	aluminium
AOT40	accumulated ozone exposure over the threshold of 40 ppb
asl	above sea level
C	carbon
CA	California
Ca	calcium
°C	degree Celsius
$CaCl_2$	calcium chloride
CH	Switzerland (Confoederatio Helvetica)
CO_2	carbon dioxide
Δ	difference
depo	deposition
df	degrees of freedom
dl	daylight
dw	dry weight
ECE	Economic commission for Europe
ED	electrochemical detector
ed.	edition
Eds.	Editors
e.g.	for example (exempli gratia)
EKL	Eidgenössische Kommission für Lufthygiene
et al.	and others (et alia)
F	charcoal-filtered
fw	fresh weight
g	gram
g	gravitational force
h	hour(s)
ha	hectare
HCl	hydrochloric acid
HNO_3	nitric acid
H_2O_2	hydrogen peroxide
H_2SO_4	sulphuric acid

IAP	Institute for Applied Plant Biology
i.e.	that is (id est)
Il	Illinois
Inc.	Incorporated
IUFRO	International Union of Forest Research Organizations
K	potassium
Karst.	Karsten
λ	wavelength
L.	Linné (Linnaeus)
l	litre
LA	leaf area
$LiSO_4$	lithium sulphate
LWC	leaf water content
M	molar
m	meter
MA	Massachusetts
MD	Maryland
Mg	magnesium
Michx.	Michaux
Mn	manganese
N	nitrogen
n	sample size
NaOH	sodium hydroxide
NH_3	ammonia
NH_4^+	ammonium
NH_4NO_3	ammonium nitrate
NITREX	nitrogen saturation experiments on forest ecosystems
NO	nitric oxide
No.	number
NO_x	nitric oxide and dioxide
NO_2	nitric dioxide
ns	not significant
NY	New York
O_2	oxygen
O_3	ozone
P	phosphorus
p	significance value
Pa	Pascal
pH	power (potential) of hydrogen
Pl	Poland
pp.	pages
ppb	parts per billion

ppm	parts per million
proj.	projected
S	Sweden
SAEFL	Swiss Agency for Environment, Forests and Landscape
SE	standard error
Se	selenium
SF	Finland (Suomi Finlandia)
U	units
UK	United Kingdom
UNECE	United Nations Economic Commission for Europe
USA	United States of America
UV	ultraviolet
v/v	volume per volume
VOC	volatile organic compounds
Vol.	Volume
W	watt
wc	water content
yr	year

10. APPENDIX

10.1 Soluble carbohydrates of *Fagus sylvatica* L.

10.1.1 Upper soil layer

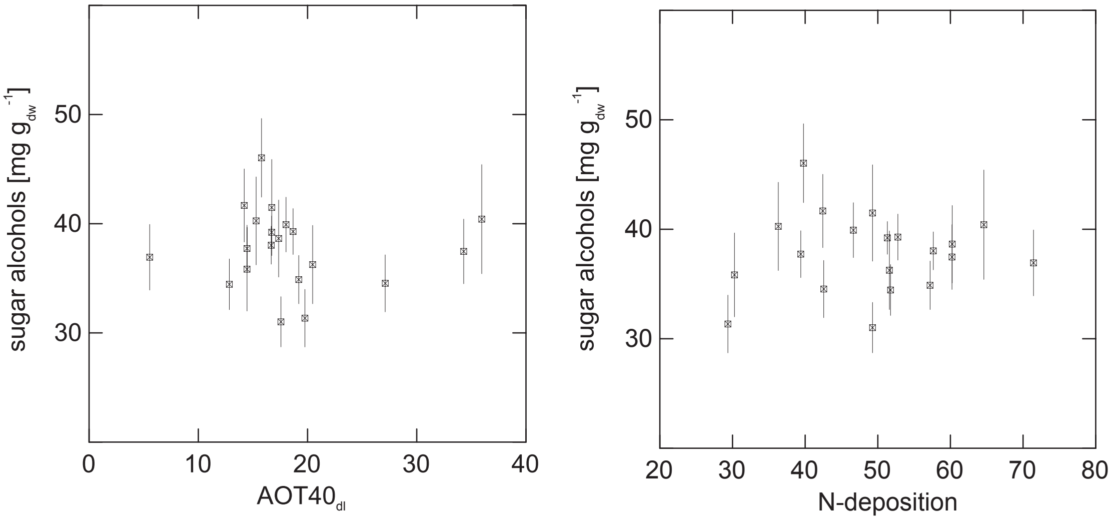

Figure 1: Sugar alcohol concentrations of fine roots of the upper soil layer along the modelled ozone (left) and nitrogen gradient (right). Ozone doses are given in ppm h > 40 ppb for the year 1999. Nitrogen deposition is given in kg N ha^{-1} yr^{-1}. Sugar alcohol concentrations are root-transformed. Observations at 20 sites, n = 124.

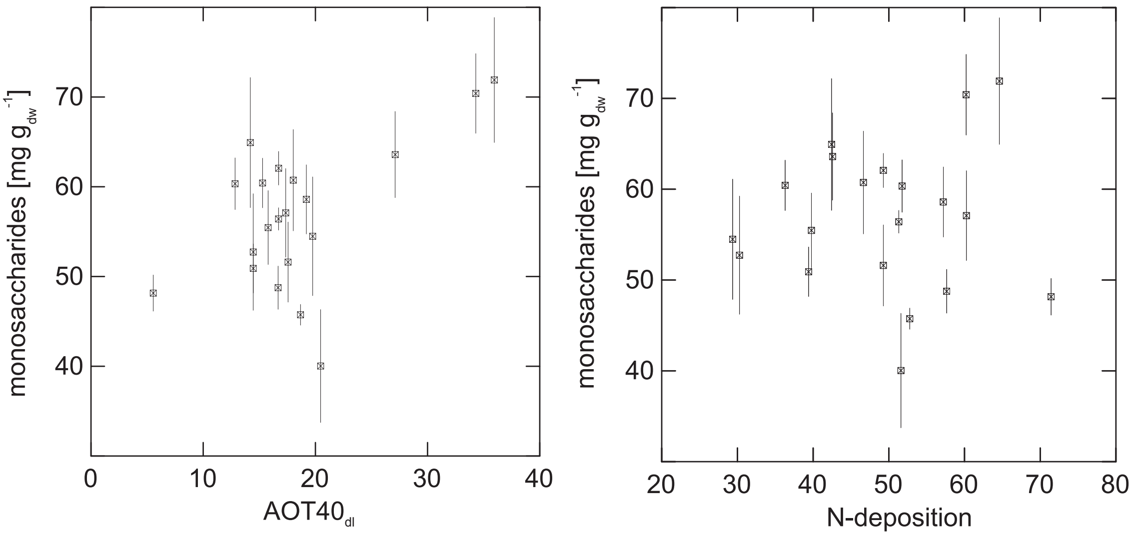

Figure 2: Monosaccharide concentrations of fine roots of the upper soil layer along the modelled ozone (left) and nitrogen gradient (right). Ozone doses are given in ppm h > 40 ppb for the year 1999. Nitrogen deposition is given in kg N ha^{-1} yr^{-1}. Monosaccharide concentrations are root-transformed. Observations at 20 sites, n = 124.

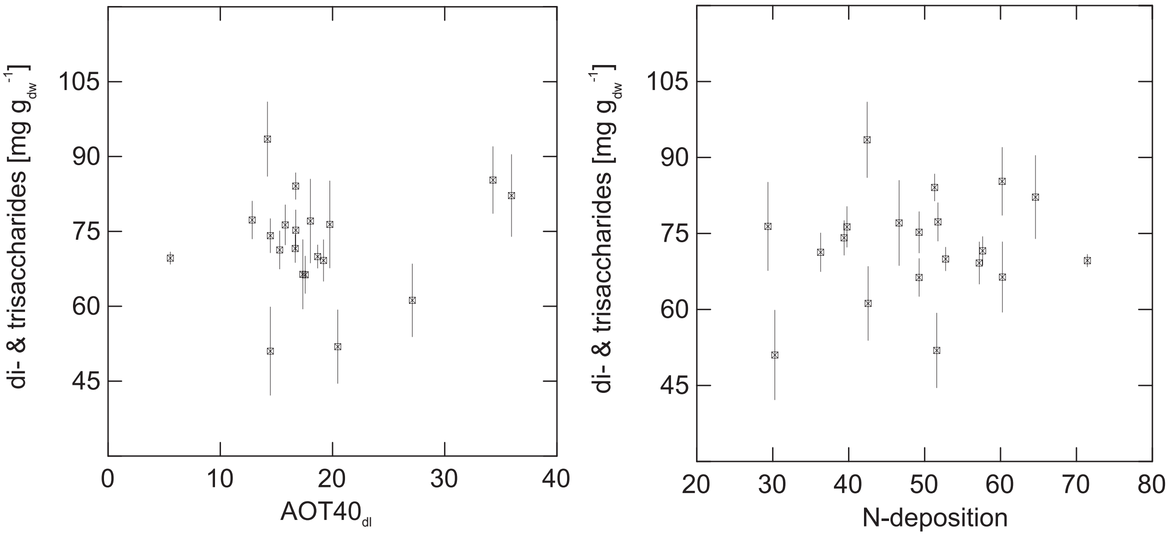

Figure 3: Di-and trisaccharide concentrations of fine roots of the upper soil layer along the modelled ozone (left) and nitrogen gradient (right). Ozone doses are given in ppm h > 40 ppb for the year 1999. Nitrogen deposition is given in kg N ha^{-1} yr^{-1}. Disaccharide concentrations are root-transformed. Observations at 20 sites, n = 124.

10.1.2 Lower soil layer

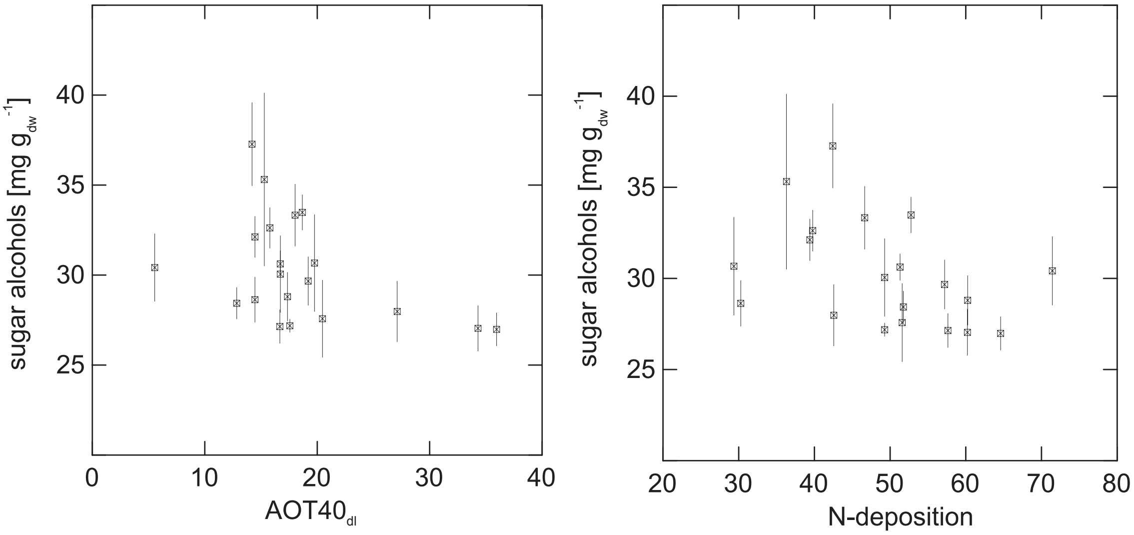

Figure 1: Sugar alcohol concentrations of fine roots of the lower soil layer along the modelled ozone (left) and nitrogen gradient (right). Ozone doses are given in ppm h > 40 ppb for the year 1999. Nitrogen deposition is given in kg N ha^{-1} yr^{-1}. Sugar alcohol concentrations are root-transformed. Observations at 20 sites, n = 124.

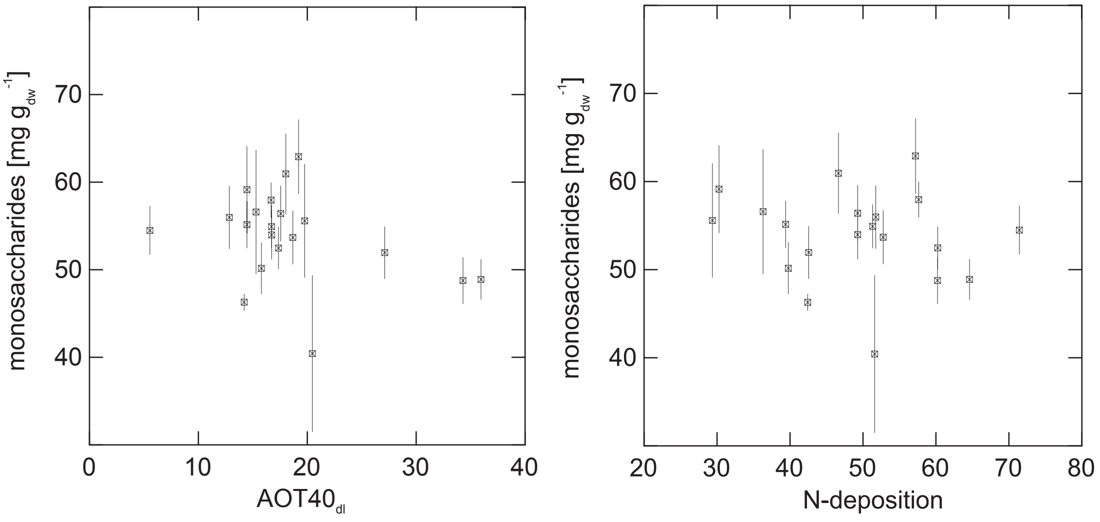

Figure 2: Monosaccharide concentrations of fine roots of the lower soil layer along the modelled ozone (left) and nitrogen gradient (right). Ozone doses are given in ppm h > 40 ppb for the year 1999. Nitrogen deposition is given in kg N ha^{-1} yr^{-1}. Monosaccharide concentrations are root-transformed. Observations at 20 sites, n = 124.

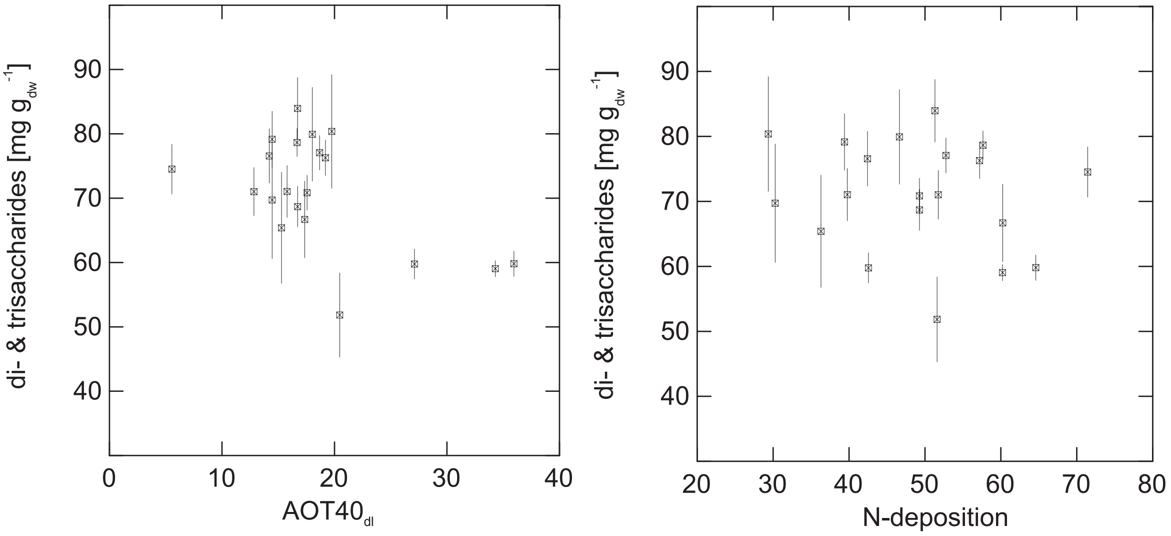

Figure 3: Di- and trisaccharide concentrations of fine roots of the lower soil layer along the modelled ozone (left) and nitrogen gradient (right). Ozone doses are given in ppm h > 40 ppb for the year 1999. Nitrogen deposition is given in kg N ha^{-1} yr^{-1}. Disaccharide concentrations are root-transformed. Observations at 20 sites, n = 124.

10.2 Soluble carbohydrates of *Picea abies* (L.) Karst

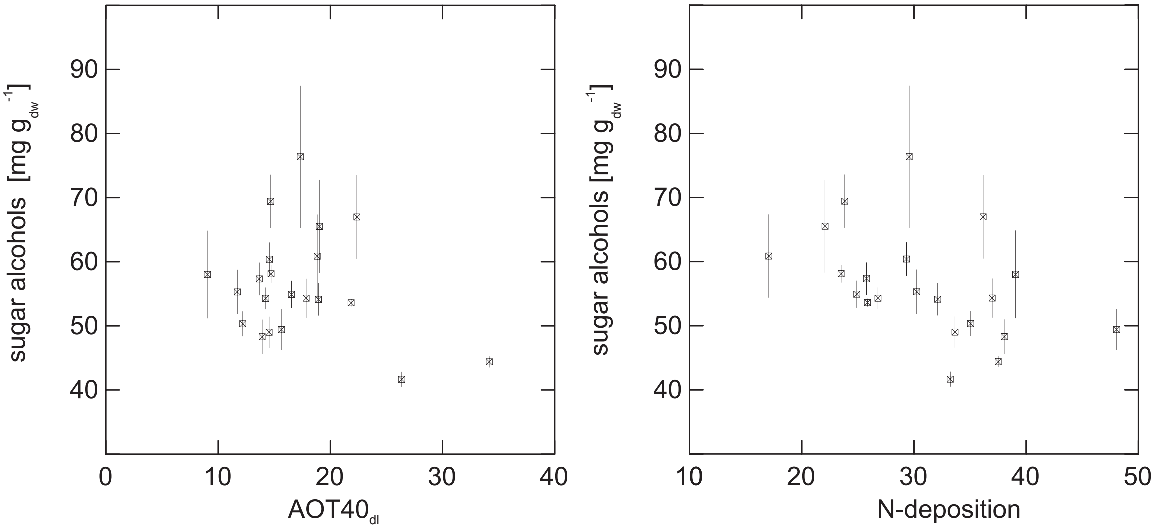

Figure 1: Sugar alcohol concentrations of fine roots along the modelled ozone (left) and nitrogen gradient (right). Ozone doses are given in ppm h > 40 ppb for the year 2000. Nitrogen deposition is given in kg N ha^{-1} yr^{-1}. Sugar alcohol concentrations are root-transformed. Observations at 21 sites, n = 126.

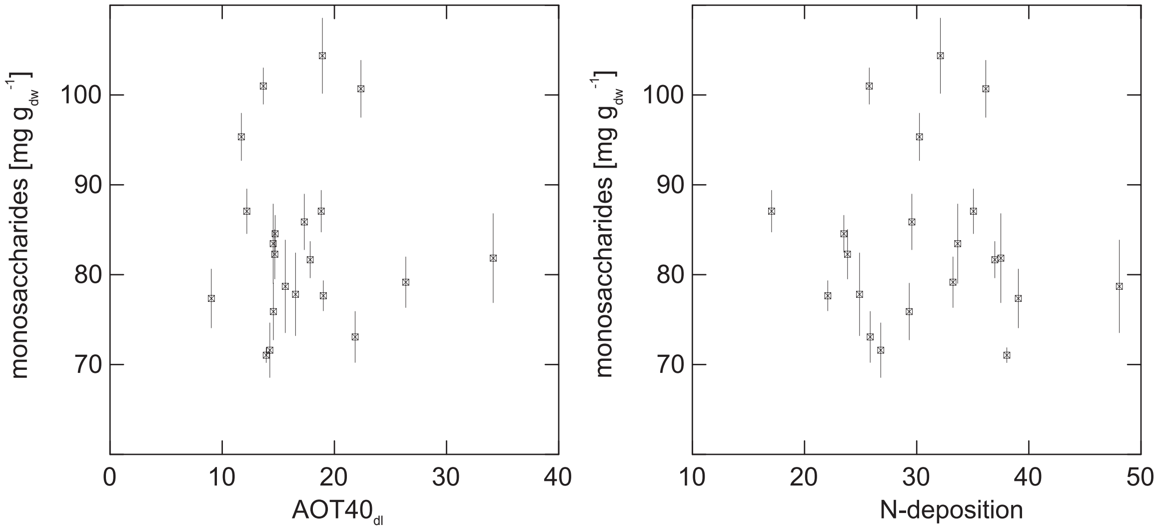

Figure 2: Monosaccharide concentrations of fine roots along the modelled ozone (left) and nitrogen gradient (right). Ozone doses are given in ppm h > 40 ppb for the year 2000. Nitrogen deposition is given in kg N ha^{-1} yr^{-1}. Monosaccharide concentrations are root-transformed. Observations at 21 sites, n = 126.

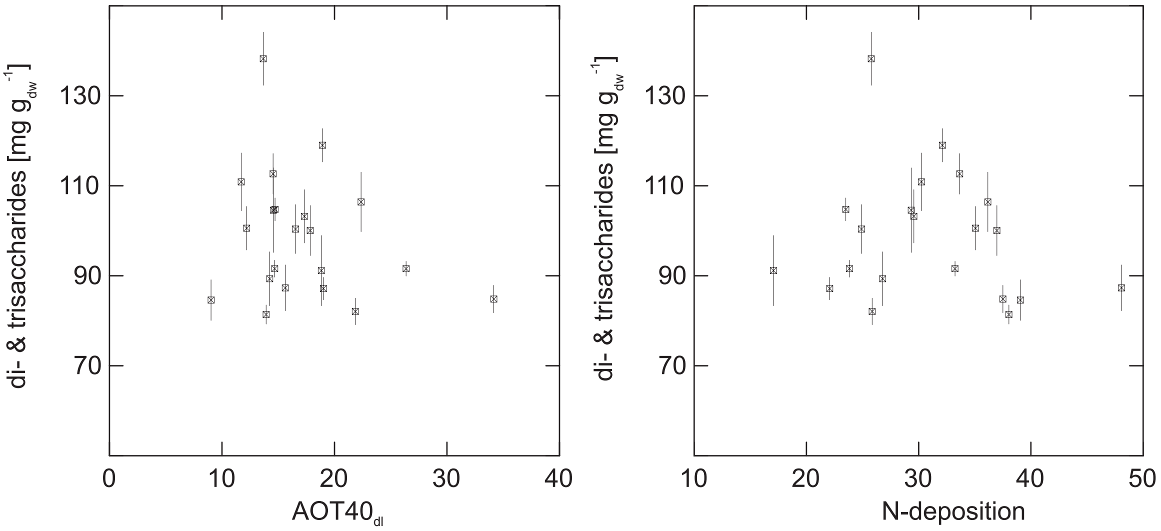

Figure 3: Di- and trisaccharide concentrations of fine roots along the modelled ozone (left) and nitrogen gradient (right). Ozone doses are given in ppm h > 40 ppb for the year 2000. Nitrogen deposition is given in kg N ha^{-1} yr^{-1}. Disaccharide concentrations are root-transformed. Observations at 21 sites, n = 126.

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12. PUBLICATIONS

12.1 Journal Articles

2005 Thomas V.F.D., Braun S., Flückiger W. Effects of simultaneous ozone exposure and nitrogen loads on carbohydrate concentrations, biomass, growth, and nutrients of young beech trees (*Fagus sylvatica*), Environmental Pollution, in press

2005 Thomas V.F.D., Braun S., Flückiger W. Effects of simultaneous ozone exposure and nitrogen loads on growth, biomass, and carbohydrate concentrations of young spruce trees (*Picea abies*), Environmental Pollution, conditionally accepted

2004 Braun S., Zugmaier U., Thomas V.F.D., Flückiger W. Carbohydrate concentrations in roots of young beech and spruce along an ozone pollution gradient, Atmospheric Environment 38: 2399 - 2407

2002 Thomas V.F.D., Hiltbrunner E., Braun S., Flückiger W. Changes in Root Starch Contents of Mature Beech (Fagus *sylvatica* L.) Along an Ozone and Nitrogen Gradient in Switzerland, Phyton 42: 223-228.

2001 Zotz G., Thomas V.F.D., Hartung W. Ecophysiological consequences of differences in plant size: abscisic acid (ABA) relations in the epiphytic orchid, *Dimerandra emarginata*, Oecologia 129: 179 - 185

1999 Zotz G., Thomas V.F.D. How much water is in the tank? Model Calculations for Two Epiphytic Bromeliads, Annals of Botany 83: 183 - 192.

12.2 Attended Conferences and Meetings

2004 Thomas V.F.D., Braun S., Flückiger W. IUFRO, Oulu (SF): Effects of simultaneous ozone exposure and nitrogen loads on growth, biomass, and carbohydrate concentrations of young spruce trees (*Picea abies*).

2003 Thomas V.F.D., Braun S., Flückiger W. British Ecological Society, Manchester (UK): Root carbohydrate contents of mature beech and spruce trees along an ozone and nitrogen gradient in Switzerland.

2002 Braun S., Zugmaier U., Thomas V.F.D., Flückiger W. UNECE workshop, Gothenburg (S): Carbohydrate concentrations in roots of young beech and spruce along a gradient of ozone pollution.

2002 Thomas V.F.D. DIONEX AG, Olten (CH) Ionchromatography in Forest Research.

2001 Thomas V.F.D., Hiltbrunner E., Braun S., Flückiger W. IUFRO, Pulawy (PL): Changes in Root Starch Contents of Mature Beech (*Fagus sylvatica* L.) Along an Ozone and Nitrogen Gradient in Switzerland.

13. CURRICULUM VITAE

PERSONAL DATA

VERA F. D. THOMAS

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Nationality: German
Date of birth: 02.05.1972
Marital status: single

EDUCATION

since 04/ 2000	Institute for Applied Plant Biology, Schönenbuch & University of Basel; PhD in Botany: „Effects of simultaneous Ozone and Nitrogen Exposure on two Tree Species: *Fagus sylvatica* L. and *Picea abies* (L.) Karst.“ (Prof. W. Flückiger, Prof. Ch. Körner)
01/ 1998 – 01/ 1999	University of Würzburg & Smithsonian Tropical Research Institute Panama; Diploma Thesis: „Investigations on Abscisic Acid in an epiphytic orchid“ (Prof. W. Hartung, PD G. Zotz) (1.3)
10/ 1992 – 01/ 1999	University of Würzburg: Diploma Studies in Biology, Main subject: Botany (1.0), Subsidiary subjects: Biotechnology (1.7), Pharmaceutical Biology (2.3)
07/ 1991	Gymnasium (high school) Miltenberg: Abitur (1.9)

SUPPLEMENTARY EDUCATIONS

10/ 2004	Training course “Project Management”: University of Zürich, Institute for Environmental Studies
06/ 2003 – 07/ 2004	WIN Mentoring Programme: Women into Industry, Resort “Chancengleichheit”, University of Basel & Novartis, Basel
08/ 1995 – 08/ 1996	Exchange studies at the State University of New York at Albany, USA
10/ 1991 – 06/ 1992	Bluefeather School of English, Dublin: Cambridge Advanced Certificate in English (A)

TRAININGS

07/ 1999 – 03/ 2000	Institute for Applied Plant Biology, Schönenbuch: Biological Training
02/ 1999 – 06/ 1999	University of Würzburg & Smithsonian Tropical Research Institute Panama: biological project work
02/ 1997 – 06/ 1997	Smithsonian Tropical Research Institute, Panama: biological field-training

JOB EXPERIENCE

since 04/ 2000	Institute for Applied Plant Biology & University of Basel *Scientific Staff* · Publications in Scientific Journals, presentations at international scientific meetings · Reviewing of scientific articles for international journals · Scientific investigations in the lab and field: analyses of soluble carbohydrates, starch, and nutrients in plant material (ion chromatography, atomabsorptionspectrometry [AAS], photometric analyses)
11/ 1994 – 07/ 1999	University of Würzburg *Scientific Staff* · Scientific coworker in the department of „Molecular Plant Physiology“ and „Ecophysiology and Vegetation Ecology“ (ELISA, gas-exchange measurements [IRGA, porometry]) · Teaching assistant, supervising students in practical lab trainings in the above mentioned departments · Tutoring for undergraduate students
Summer Jobs	· Spices- and Teatrade „Wild" 1994 – 1999 (sales assistant) · Terrace-Cafeteria, Würzburg (waitress) · Road Maintenance Office, Miltenberg (secretary) · Touristic Shipping Company, Miltenberg (waitress) · Private tutor for school children (English), Miltenberg

AWARDS & SCHOLARSHIPS

2004	Award for outstanding poster presentation at the IUFRO-meeting, Oulu (SF)
1998	Scholarship for exchange studies in Panama (German Academic Exchange Office)
1996	Scholarship for books and study materials (Karl-von-Foster-Scholarship, University of Würzburg)
1995	Scholarship for State University of New York at Albany (University of Würzburg, granted)
1991	Award for outstanding Abitur

LANGUAGES

German	Native language
English	Fluent in speaking and writing, several stays in English speaking countries, Scientific English
French	Knowledge acquired at school and evening classes

HONORARY POSTS

Since 2002	"Grüne Schule" Basel, Guided tours for pupils, Botanical Garden, Basel
1992-1995	Active member of Students Department of Biology, Würzburg
1987-1991	Joint Administration by Pupils, Spokesperson of pupils at high school, Miltenberg
1988-1990	Leader of youth group, Protestant Community, Miltenberg

PROFESSORS & ASSISTANT PROFESSORS (BASEL)

Prof. Dr. W. Flückiger, Prof. Dr. Ch. Körner, Dr. E. Lüdin, Dr. F. Schweingruber, PD Dr. J. Stöcklin, Dr. V. Wiemken, PD Dr. G. Zotz